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ANTIBODIES SPECIFICALLY RECOGNIZING A NITROSYLATED PROTEIN, THEIR METHOD OF PREPARATION, THERAPEUTIC USE AND DIAGNOSIS.

5   CLAIM OF PRIORITY
          This application is a 35 U.S.C. 371 national application of PCT/FR97/02412, filed on December 23, 1997. This application also claims priority to French Patent Application FR 96/16207.

10   TECHNICAL FIELD OF THE INVENTION
                    The present invention concerns antibodies specifically recognizing a nitrosylated protein, particularly compounds resulting from the conjugation of NO with its carriers, such as albumin. The invention also relates to immunogenic compounds for the preparation of such monoclonal or polyclonal antibodies. The invention also involves
15   pharmaceutical compounds which include these antibodies as active ingredient, and their use in the treatment of pathologies involving nitric oxide, its derivatives and conjugates, in particular situations in which there is an excessive production of NO. Finally, the invention concerns the use of these antibodies for the detection of nitrosylated compounds and the diagnosis of pathologies involving these compounds.
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                    Nitric oxide, hereinafter designated also as NO, is described as being the smallest molecule made by the cells. Initially assimilated to endothelium derived relaxing factor (EDRF), it was then recognized as a neuromediator, and is thought to be the first neurotransmitter with retrograde activity, as well as a cytostatic/cytotoxic molecule.
25   Because of its strong reactivity, nitric oxide is capable of reacting with a large number of molecules to form conjugates which have multiple functions and therefore participate in many physiological and pathophysiological processes.

                    Nitric oxide produced in large quantity or in insufficient amounts is involved
30   in a large number of pathophysiological processes, such as infections, septic shock, degenerative and autoimmune diseases, graft rejection, and its action is often relayed by that of its derivatives and conjugates. Thus, nitrosylated albumin possesses hypotensive activity, which explains the duration of action of the NO. Likewise, nitrohemoglobin has recently been described as acting on vasodilation.

In order to compensate for the harmful effects of excessive or inadequate production of NO or its conjugates, former methods have proposed the use of inhibitors of the enzyme NO-synthase. However, it was noted that these inhibitors are not active on already formed molecules and that, moreover, their use is limited by their distribution in the

5   body and their toxicity.

SUMMARY OF THE INVENTION

The present invention aims specifically to compensate for these disadvantages by proposing biological molecules acting selectively on the molecules

10   carrying nitric oxide.

In addition, the detection of nitrosylated substances would permit the discovery of molecules or target tissues involved in the previously cited pathologies. Former methods proposed, therefore, the use of anti-nitrotyrosine antibodies to reveal the

15   nitrated tyrosine, which is the marker for the formation of peroxynitrite, resulting from the reaction between the superoxide anion O_2^- with NO, in numerous pathologies.

The present invention also aims to provide new methods for the detection of nitrosylated molecules useful in the diagnosis of pathologies in which they are involved.

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These goals are attained according to the invention with purified antibodies specifically recognizing a nitrosylated protein, and particularly compounds resulting from the conjugation of NO with its carriers. Of these, the invention concerns more specifically purified antibodies directed against nitrosylated albumins. In the following, nitrosylated

25   protein refers both to nitrosylated peptides and polypeptides and more generally all nitrosylated antigenic conjugates.

The antibodies of the invention present numerous advantages, in particular relative to their rapidity of action and their efficacy on already formed and active

30   nitrosylated molecules. In addition, their ability to diffuse in the body is limited to the physicochemical properties of immunoglobulins. Aside from their efficacy, the antibodies of the invention have the advantage of being very specific and non-toxic.

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DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph illustrating the zone of absorbency of NO-Tyr-BSA.

Fig 2 is a graph illustrating the zone of absorbency of NO-Cys-BSA.

Fig. 3 is a graph illustrating response following immunization with NO-Tyr-BSA.

Fig. 4 is a graph illustrating response following immunization with NO-Cys-BSA.

Fig. 5 is a graph illustrating the avidity of conjugated anti-NO-Tyr Ab and conjugated anti-NO=Cys in competition tests,

Fig. 6 illustrates the kinetics of the formation and concentration in NO-Cys-BSA formed in supernatant of the culture of activated macrophages determined at incubation times: 0, 3, 4, 6, 8, 11, 14, 18 and 20 hours using "C" antiserum.

Fig. 7 illustrates inhibition of the cytostatic effect of the BCG macrophages on the *T. musculi in vitro* in the presence of NMMA (0.5 mM), ("T") or ("C") antiserum used at 1/100.

Fig. 8 illustrates the cytostatic effect of supernatants containing NO-BSA from activated macrophages added to normal macrophages containing *T. musculi.* Inhibition of this effect in the presence of NMMA (0.5 mM), ("T") or ("C") antiserum used at 1/100.

Fig. 9 illustrates the spectrometric analysis of the NO-Cys-G-BSA immunogen and its structural homologue Cys-G-BSA based on wavelength.

Fig. 10 illustrates the evolution of the antibody response during immunization of the intraperitoneally immunized mouse.

Fig. 11 illustrates the evolution of the antibody response during immunization of the intraperitoneally immunized mouse.

Fig. 12 illustrates the avidity and specificity of the anti-NO-Cys-G antibodies in the mouse (IP).

Fig. 13 illustrates represents the avidity and specificity of the anti-NO-Cys-G mouse antibodies (PC).

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Fig. 14 illustrates the avidity and specificity of the anti-NO-Cys-G monoclonal Ab.

Fig. 15a is a high-magnification (100X) immunocytochemical marking illustrating anti-NO-Cys-G monoclonal antibody and showing very clear markings (immunoreactivities) in terms of trypanosomes co-cultivated in the presence of the activated macrophages.

Fig. 15b is a high-magnification (100X) immunocytochemical marking illustrating a much weaker marking obtained in the co-culture of activated macrophages / trypanosomes, in the presence of NMMA (0.5 mM).

Fig. 15c is s a high-magnification (100X) immunocytochemical marking illustrating a total absence of trypanosome marking was obtained when a normal mouse serum was used.

Fig. 15d is a high-magnification (100X) immunocytochemical marking illustrating antibody "C" having an intensity very close to the one for the monoclonal Ab.

Fig. 15e is a high-magnification (100X) immunocytochemical marking illustrating the anti-NO-Tyr ("T") giving a positive marking with an intensity not as high as the two types of antibodies (monoclonal and polyclonal) directed against the epitope NO-Cys.

Fig. 15f is a high-magnification (100X) immunocytochemical marking illustrating the absence of marking in the primary Ab of a normal rabbit.

Fig. 16 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-BSA epitope.

Fig. 17 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on delipidated NO-BSA epitope.

Fig. 18 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-Cys-BSA epitope.

Fig. 19 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-Cys-G-BSA epitope.

Fig. 20 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-Tyr-BSA epitope.

Fig. 21 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-Tyr-G-BSA epitope.

Fig. 22 illustrates the OD values for the normal mouse serum and the parasitized mouse serum showing the responses on NO-Tryp-G-BSA epitope.

Fig. 23 illustrates the OD obtained from ELISA tests on the NO-Cys-G-BSA, NO-Tyr-BSA and NO_2-Tyr-BSA conjugates (the control group) and indicating presence of circulating Ab whose rate increases during attacks an ddecreases during remissions.

Fig. 24 illustrates the OD obtained from ELISA tests on anti-NO-Tyr-BSA and anti-NO2-Tyr-BSA conjugates (the aminoguanidine group).

Fig. 25 illustrates the OD obtained from ELISA tests on anti-NO-Cys-G, anti-NO-Tyr and anti-NO_2-Tyr and showing the changes over time in the anti-NO-Cys-G, anti-NO-Tyr and anti-NO_2-Tyr responses (the monoclonal antibody group).

Fig. 26 illustrates the progression of antibodies directed against NO-Cys-G-BSA, NO-Tyr-BSA and NO2-Tyr-BSA conjugates (the control group).

Fig. 27 illustrates the progression of antibodies directed against NO-Cys-G-BSA, NO-Tyr-BSA and NO2-Tyr-BSA conjugates (the NIS group).

Fig. 28 illustrates the progression of antibodies directed against anti-NO-Tyr-BSA and anti-NO2-Tyr-BSA conjugates (the aminoguanidine group) ; and

Fig. 29 illustrates the progression of antibodies directed against anti-NO-Cys-G, anti-NO-Tyr and anti-NO_2-Tyr conjugates (the monoclonal antibody group).

DETAILED DESCRIPTION OF THE INVENTION

Antibodies are macromolecules made by the body in response to the presence of a foreign substance. Antibodies of animal origin have been used for a long time

in human therapy. For example, horse serum has been used as an anti-tetanus antibody following immunization with the tetanus toxin.

The research leading to the present invention began with the induction of polyclonal antibodies directed against a nitrosylated protein, which is usually more stable *in vivo* than a nitrosylated amino acid. In addition, the different immune responses were studied by modifying the animal species in addition to the epitopes and their presentation. The invention also concerns a group of previous polyclonal antibodies obtained by injecting an animal with an immunogen described below. Monoclonal antibodies were then prepared in accordance with the traditional methods of cellular fusion.

NO has a great affinity for cysteine and tyrosine and for binding with them. It is involved in multiple functions which may be related to processes of transport, storage, and finally release of NO at its sites of action.

Polyclonal and monoclonal antibodies were made using different nitrosylated antigenic molecules. To make them immunogenic, the amino acids (haptens) were coupled with carrier proteins using different coupling agents. Thus albumin, which is the most abundant plasma protein, stores and transports NO, and it has been demonstrated that this nitrosylated albumin possesses significant vasodilating as well as cytotoxic and cytostatic activity. This property of albumin is due to the presence of a cysteine with increased affinity for NO. Research work carried out by the Inventors and reported below permitted the preparation of poly- and monoclonal antibodies directed against NO conjugates and amino acid radicals, cysteine, tyrosine and tryptophan, which are themselves coupled with a carrier protein, human or bovine serum albumin. The specific antibodies of the nitrosylated serum albumin were then selected and used to recognize and neutralize certain of the *in vitro* properties of this conjugate.

By synthesizing several forms of immunogens, the Inventors demonstrated that these compounds are sufficiently stable to induce an immune response and that this response is specifically directed against the corresponding nitrosylated epitope. The possibility of inducing these antibodies and the intervention of nitrosothiols and cysteine in the vasodilation processes (Ignarro et al., 1981) led the Inventors to research the existence of *in vitro* and *in vivo* nitrosylated derivatives and to block their effects.

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The invention also concerns immunogens for the preparation of the antibodies defined above. These immunogens are comprised of a nitrosylated amino acid coupled with a carrier protein. An example of the synthesis of these immunogens is the coupling of the L-tyrosine hapten (Tyr) or acetylated L-cysteine-N (Cys) with a carrier protein such as bovine serum albumin (BSA) or human serum albumin (HSA) with carbodiimide. The conjugates obtained are then nitrosylated with an NO carrier such as sodium nitrite (NaNO$_2$) in an acid medium. The use of different coupling agents such as carbodiimide, glutaraldehyde (G) or succinic anhydride (SA) and proteins, peptides or distinct carrier polypeptides yield epitopes with different conformations associated with the nitrosylation.

These immunogens may also be composed of a protein with a sequence containing an amino acid radical capable of being nitrosylated such as albumin, which contains a cysteine.

In the immunogens of the above invention, carrier protein is used to refer to both peptides and polypeptides.

The following nitrosylated conjugates were prepared within the context of the invention: NO-Tyr-BSA; NO-Cys (acetylated)-BSA; NO-Cys; (non-acetylated)-BSA; NO-Tyr-G-BSA; NO-Cys-G-BSA; NO-Tyr-SA-BSA; NO-Cys-SA-BSA; NO-Tryp-BSA; NO-Tryp-G-BSA; NO-BSA. The preparation of these nitrosylated antigens permitted the development of poly- and monoclonal antibodies which were used to detect the nitrosylation of bodily molecules (BSA) or pathogenic agents and to neutralize their activity. The production of Ab specifically targeted against immunogens carrying a nitrosylated epitope is delicate, since numerous couplings of different small molecules with carrier proteins proved effective in one animal species, but not in another. This fact was noted during modification of the antigen presentation. In the rabbit, for example, two immunogens proved effective, NO-Cys (acetylated)-BSA and NO-Tyr-BSA, in which the coupling agent used was carbodiimide. The development of these rabbit immune serums permitted development of murine monoclonal antibodies. These murine antibodies first required the production of polyclonal Ab in the mouse, in which the coupling with glutaraldehyde was the only one which yielded good results. These results were only

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obtained when the immunogen NO-Cys-G-BSA was used. The answers obtained relative to the other conjugates were not specific to the nitrosylated epitope. As a result, the *in vivo* presentation of the immunogen proved to be a determining element for stimulating the immune response, and obtaining a successful approach against small nitrosylated

5   molecules.

Research work performed by the Inventors led to demonstration of the activities carried by the nitrosylated molecules and their neutralization with antibodies directed against nitrosylated epitopes. As a result, the invention concerns pharmaceutical

10   compounds that include an antibody from the invention in particular as an active ingredient dispersed in pharmaceutically acceptable fillers or excipients. The invention also relates to the use of antibodies of the invention for the preparation of medications used to treat or prevent pathologies which involve NO, its derivatives or conjugates, such as infection by microorganisms and parasites, autoimmune and inflammatory diseases, septic shock,

15   cancers, graft rejection, and neurotoxicity. Overproduction of "bound-NO" is thought to be responsible for the deleterious effect of NO in certain pathologies, and could therefore be neutralized by antibodies of the invention specifically and previously humanized.

The Inventors demonstrated *in vivo* the role of NO in the development of

20   autoimmune and inflammatory diseases. Until the present, inhibitors of NO-synthase were the molecules most often used to block the activity of NO in given pathological conditions. The research carried out within the context of the present invention on two experimental diseases, experimental autoimmune encephalitis and inflammatory arthritis in the female Lewis rat, permitted demonstration of the usefulness of the antibodies of the invention as

25   inhibitors of the harmful effect of NO or its derivatives.

The antibodies of the invention are extremely useful physiologically and pathophysiologically in both therapeutic and diagnostic applications. They permit detection of the synthesis of molecules carrying NO-cysteine epitopes and therefore clarification of

30   new mechanisms and treatment of illnesses involving nitric oxide, its derivatives and conjugates, in particular in situations which involve excessive production of NO, such as infections, shock, acute or chronic infections (systemic or localized), transplants, degenerative diseases, diabetes, autoimmune diseases, cancers, etc.

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The invention also concerns a procedure for *in vitro* detection of nitrosylated proteins in a biological sample, such as fluid or tissue, including at least the following steps:

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- placement of this sample in contact with at least one antibody of the invention or a group of these antibodies, possibly labeled, in conditions permitting the formation of immunological complexes;
- the detection of an antigen-antibody immunological complex using physical or chemical methods.

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The invention also concerns a kit for the *in vitro* detection of nitrosylated proteins in a biological sample, in particular for the diagnosis of pathology involving NO, its derivatives or conjugates. This kit includes:

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- at least one antibody of the invention or a group of these antibodies possibly labeled;

- reagents to establish a medium hospitable to the immunological reaction between said antibody and the nitrosylated proteins possibly present in a biological sample;

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- if possible, one or several labeled detection reagents possibly labeled capable of reacting with the immunological complexes possibly formed;

- if possible, one or several biological reference or control reagents.

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Other advantages and characteristics of the invention will appear in the following examples concerning:

- characterization and functions of NO;

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- preparation of polyclonal antibodies directed against nitrosylated antibodies;

- detection and neutralization of NO derivatives with polyclonal antibodies;

- preparation of monoclonal antibodies directed against NO-cys-glutaraldehyde conjugates coupled with a carrier protein;

5   - detection of nitrosylated antigens and the protective role of monoclonal antibodies.

I - CHARACTERIZATION AND FUNCTION OF NITROGEN MONOXIDE

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Nitrogen monoxide or nitric oxide is a diatomic gas radical. It is produced enzymatically by several isoforms of the No-synthase enzyme. NO synthesis takes place in numerous cell types. This explains in part its involvement in a very large variety of biological functions (Moncada et al., 1991; Knowles and Moncada, 1992; Lowenstein and
15   Snyder, 1992; Nathan, 1992; Stamler et al., 1992a).

a) NO synthesis:

Formation of NO takes place by oxidation of L-arginine in the terminal nitrogen of the guanidine function. Production of NO from arginine leads to the
20   simultaneous formation of L-citrulline (Marletta et al.,1988).

Oxidation of L-arginine is controlled by NOS and requires the presence of molecular oxygen and cofactors such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH4), nicotinamide adenine dinucleotide
25   phosphate (NADPH) (Marletta, 1993; 1994).

b) Different forms of NO-synthase (NOS):

Several types of NOS were cloned and classified in two distinct families:
30   NOS termed constitutive (cNOS) or inducible NOS (iNOS).

A fundamental difference between these isoforms concerns the NO synthase activity *in situ* (Bredt et al., 1991; Mondaca et al., 1991; Hevel and Marletta, 1992; Lamas et al., 1992; Nathan, 1992): The cNOS are calcium /calmodulin dependent and produce

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only slight quantities of NO (picomoles/min/mg protein) in short periods of time in response to receptor activation in physiological conditions (Stuehr and Griffith, 1992). These enzymes are generally found in endothelial cells, neurons, astrocytes, platelets, neutrophil leukocytes and adrenal glands (Moncada et al., 1991; Lacaze-Masmonteil, 1992; Nussler et al., 1995). The iNOS are calcium /calmodulin independent. They are induced by immunocompetent cells, primarily by cytokines, and numerous other molecules, in particular derivatives of microorganisms (Moncada et al., 1991). Stimulation of iNOS leads to the synthesis of large quantities of NO (nanomoles/min/mg protein) for long periods (Nathan and Xie, 1994). These discharges of NO are responsible for cytostatic and cytotoxic phenomena. These iNOS are found in activated macrophages, muscle cells, hepatocytes, fibroblasts, astrocytes, neutrophil leukocytes and endothelial cells (Stuehr and Griffith,1992; Marletta, 1993; Anggard, 1994).

In man, an iNOS was demonstrated in the hepatocytes and smooth muscle cells of the aorta (Geller et al.,1993), in chondrocytes (Palmer et al., 1993), keratinocytes (Heck et al., 1992), astrocytes (Lee et al., 1993) and the islets of Langerhans where induction of NOS by interleukin 1 (IL-1) may participate in the destruction of these cells (Corbett et al., 1993). More recently, NO production was demonstrated in human monocytes by a transduction mechanism involving CD23 (Dugas et al., 1995).

c) Inhibitors of NOS:
Numerous arginine N-substitutes were synthesized as specific NOS inhibitors, including: Nco monomethyl-Larginine (NMMA) which is a good inhibitor of three isoforms and Nco-nitro L-arginine which is more active on cNOS (Stuehr and Griffith, 1992). Aminoguanidine is more active on the iNOS (Misko et al., 1993). Other inhibitors of different NOS isoforms have recently been demonstrated such as thiocitrullin (Southan et al., 1995) and 7-nitro-indazol (Mayer et al., 1994).

d) The chemistry and biochemistry of NO:

In contrast to most free radicals, nitric oxide does not dimerize or dismutate. Its reactivity depends primarily on its capacities to yield its lone electron to other radicals (superoxide ion, tyrosyl radical, etc.) or to species capable of intervening in radical reactions (molecular oxygen, thiols, phenols, etc.) or to transitional metals absorbent of

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electrons (iron, copper...) (Stamler et al., 1992a). NO is either made into a complex, or transformed into NO+ nitrosonium ion. The potential for oxidation-reduction of the surrounding medium determines the transformation of NO to NO+ and NO-. However, two ions have never been proved to exist in physiological mediums.

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- Reaction of NO with oxygen:

NO is oxidized in nitrite (NO_2^-) in the presence of oxygen (Farkas and Menzel, 1995). Its life cycle *in vivo* depends primarily on this reactivity with oxygen. In the

10   gas state, the radical nitric dioxide ($NO_2°$) is formed, which dimerizes into N_2O_4 and dissolves in water yielding nitrous and nitric acid. In the dissolved state and with a pH of 7.4, the mixture of NO° and oxygen generates only NO_2-, but not nitrate, with the following stoichiometry:

15            $$4NO + O_2 + 2H_2O \text{ --------> } 4HNO_2 \text{ -------> } 4H^+ + 4NO_2$$
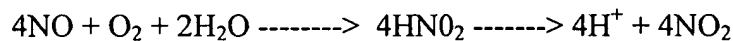
- Reaction with the superoxide anion:

The derivatives of oxygen, superoxide ion ($O_2°$), the hydroxyl radical (HO°)

20   and oxygenated water (H_2O_2) are generated in normal and pathological metabolic processes. Simultaneously with NO, the superoxide ion $O°^{2-}$, produced by NADPH oxidase, is secreted by various tissues, particularly during inflammations and septic shock. The coupling of NO and $O°^{2-}$ radicals yields peroxynitrite with a reaction constant of 6.7 x 10^9 mol^{-1} L s^{-1}.

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In fact, the reaction constant of SOD with $O_2°^-$ being (2 x 10^9 mol^{-1} Ls^{-1}) NO and superoxide dismutase (SOD) are in competition to trap O_2- (Koppenol et al., 1992). Peroxynitrite can also be formed by the reaction of NO_2- with H_2O_2 or the nitroxyl anion (NO-) with oxygen (Fontecave and Pierre, 1994; Butler et al., 1995):

30            $NO° + O_2°-$
             $NO_2- + H_2O_2 \text{ =======> } O=N-O-O \text{ ----> } NO_3^-$
             $NO^- + O2$
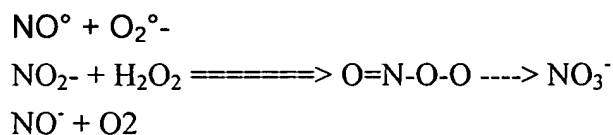
- Reaction with hydrogen peroxide:

H_2O_2 can react directly with NO. Its concentration in biological mediums is greater than the concentration of $O_2^{-\circ}$ (Fontecave and Pierre, 1994). The reaction does not yield ONOO- but rather the oxygen singlet ($^1O_2$) detectable with chimioluminescence:

$$2NO + H_2O_2 \longrightarrow {}^1O_2 + H_2O + N_2O$$
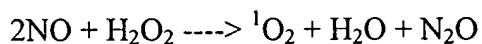
This oxygen singlet formed is very reactive and can participate in cellular destruction and inflammatory processes during macrophage activation.

-Reaction with thiols:

The thionitrites, such as S-nitrosocystein or nitrosoalbumin, with a longer life than NO, are compounds likely to transport it from NOS to the NO target.

The thionitrites can be formed by nitrosylation from free thiols present in the cellular cytosol and blood (Girard and Potier, 1993). Formation of thionitrites following activation of NOS *in vivo* has been described many times: it may play a role in the modulation of certain enzymatic activities as well as in NO transport.

Numerous works have demonstrated the nitrosylation of the free SH grouping of free cysteine or bovine serum albumin (Cys 34). It was recently demonstrated that NO released in a biological system reacts in the presence of thiols to form S-nitrosoprotein derivatives. In fact, the plasma proteins serve as an NO reservoir produced by the endothelial cells (Stamler et al., 1992b).

In man, plasma contains approximately 7 μM S-nitrosothiols, 96% of which are in the form of S-nitrosoproteins, 82% of which are S- nitro-serum-albumin (Stamler et al., 1992b). The plasma concentration of thiols is 0.5 mM and plasma concentration of NO is 3 nM (Stamler and al., 1992b). The free NO has a half-life of several seconds to several minutes (Kelm and Schrader, 1990). In plasma, in the SNO-Cys or S-NO-protein form, it has a longer half-life, respectively 10 and 40 min (Ignarro et al., 1981; Ignarro, 1989; Stamler et al., 1992c). In a phosphate buffer with a pH of 7.4 at 25°C, NO-BSA has a half-life of approximately 24 hours (Stamler et al., 1981, 1992c, d) which decreases to 12 hours

at 37°C (Stamler et al., 1992c). Under these same conditions, NO and S-nitrosocystein have respective half-lives of 0.1-1s (Kelm and Schrader, 1990) and 15-30 s (Ignarro et al., 1981).

In vitro, the treatment of thiols in an acid medium with a nitrosating agent
5   such as $NaNO_2$, yields the following reaction (Fontecave and Pierre, 1994):

$NaNO_2$,

RSH ----> R^1SNO (nitrosothiol)
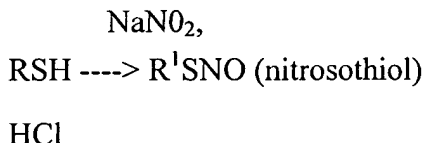
HCl
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Formed nitrosothiol can transfer NO to a second thiol or another nucleophile through the mechanism of transnitrosylation (Butler et al., 1995):

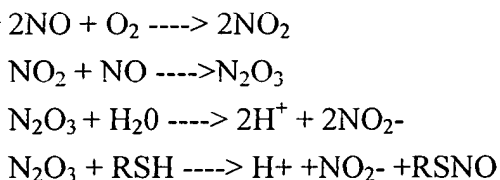$R^1SNO + R^2S^- \text{-------} > R^1S^- + R^2SNO$
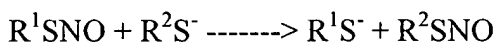

15   <u>- Reaction with serum albumin:</u>

At a pH of 7, NO does not react directly with the thiols to yield nitrosothiols; their formation may be observed in an oxygenated medium. Recent data demonstrate that for thiols of low molecular weight (N-acetyl-Cys, and glutathione), the essential nitrosating agent is N_2O_3 (Kharitovov et al., 1995) according to the following reactions:

20       $2NO + O_2 \text{----}> 2NO_2$

$NO_2 + NO \text{----}>N_2O_3$

$N_2O_3 + H_2O \text{----}> 2H^+ + 2NO_2-$

$N_2O_3 + RSH \text{----}> H+ +NO_2- +RSNO$

25   On the other hand, in the case of the two human and bovine serum albumin proteins the situation is different because the speed of the reaction is 10 times less than that of low molecular weight thiols ($3-1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, compared to $0.3-0.06 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$). Their nitrosylation by N_2O_3 is therefore not significant. Keaney et al., (1993) suggested the possibility of nitrosylation of HSA by its reaction with NO^+ which could occur following
30   the reaction of NO with Fe^{2+} of hemoproteins such as hemoglobin (Kharitonov et al., 1995):

$Hb^+ NO \text{----}> Hb + NO^+$

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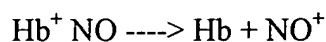
- Reaction with tyrosine:

Since tyrosine has an aromatic group, nitrosylation occurs by the transfer of charges between NO+ and this aromatic group which yields an electron:

5          $Ar-NO+ \Longleftrightarrow Ar^{\circ} + NO$ (Stamler et al., 1992a).
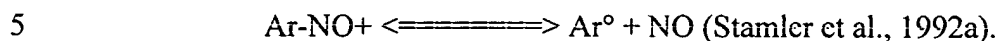
This reaction may take place in a protein containing an amino acid radical in its active site. This is the case with the reductase ribonucleotide (Fontecave and Pierre, 1994). In the R_2 sub-unit of this enzyme, there is a stable tyrosyl radical which is essential

10   for enzymatic activity. The reversible reaction of this radical with NO may explain in part its regulating mechanism in the enzyme.

- Reaction with transition metals:

15   Numerous NO targets have proved to be metalloproteins. In fact, NO° can bind to all transition metals. It is used, therefore, as an inhibitor of transport proteins and oxygen metabolism: hemoglobin, myoglobin, oxydases, oxygenases, etc... The affinity of NO is generally much stronger for Fe^{2+} than for Fe^{3+}. NO also binds to Co^{2+} in some of its states of oxidation, and to Mn^{2+} and Cu^{2+}. NO can also act as a reducing agent vis-à-vis

20   certain metalloproteins (Henry et al., 1991; Stamler et al., 1992a; Traylor and Sharma, 1992; Henry et al., 1993).

e) Physiological and pathological roles of NO:
- Role of NO in the cardiovascular system:

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In 1980, Furchgott and Zawadski noted that relaxation of isolated arteries subject to the action of acetylcholine depends upon the endothelium (Furchgott and Zawadski, 1980). They deduced from this fact the existence of a fleeting factor called "endothelium derived relaxing factor" (EDRF), secreted by the endothelial cells treated

30   with acetylcholine or bradykinine, leading to the elongation of adjacent smooth muscle cells. Its chemical nature was only deciphered in 1987 by Palmer and Mondaca (Palmer et al., 1987, 1988; Ignarro et al., 1987).

In addition to the EDRF action, radical NO exerts an effect on the platelets: the increase in the GMPc under its control causes a decrease in platelet aggregation and adhesion (Radomski et al., 1987). In man NO is used for therapeutic purposes: molsidomine, for example, is an NO donor used as a vasodilating agent in the treatment of a certain number of cardiovascular disorders.

NO is also used directly in inhalation in a gaseous form at a dose of 10 to 40 p.p.m. in certain resuscitation services, with the goal of causing vasodilation of the pulmonary circulation, and therefore improving respiratory gas exchanges in the treatment of patients suffering from severe pulmonary hypertension or the acute respiratory distress syndrome in adults (ARDS) (Pepke-Zaba et al., 1991; Falke et al., 1991).

Massive NO production

An excess of NO production can have a harmful effect on the cardiovascular system. In septic shock, for example, a massive amount of NO is secreted not only by the endothelial cells but also by the mastocytes, smooth muscle fibers, leukocytes and renal cells. This significant release of NO is due to iNOS induction (Anggard, 1994). The excess of synthesized NO is responsible for hypotension, vascular hyporeactivity and myocardial depression (Lancaster, 1992) in experimental septic shock induced by endotoxin or cytokines (Kilbourn et al., 1990; Reed et al., 1990; Thiemermann and Vane, 1990; Gray et al., 1991; Vallance and Moncada, 1993) and may cause tissue destruction (Palmer et al., 1992).

In man, it has been demonstrated that use of NMMA in patients with septic shock leads to a dose-dependent increase in blood pressure (Petros et al., 1994). These results indicate NO's contribution to cardiovascular changes and suggest a possible role played by NOS inhibitors in septic shock therapy.

Decreased production of NO

A defect in NO production could be the root cause of some cases of increased blood pressure. In the genetically hypertensive salt-sensitive rat (Dahl rate),

administration of L-arginine lowers blood pressure (Chen and Sanders, 1991). This decrease in blood pressure may be the result of stimulation of NO production.

5   In experimental models of atherosclerosis, a reduction in NO release by the vascular endothelium in rabbits has been demonstrated (Coene et al., 1985). It is possible that the destruction of the endothelium, in particular by atherosclerosis, leads to an inability to correctly synthesize NO, which causes a decrease in vasorelaxation and perhaps a permissive effect on cellular proliferation.

10   Therefore, hypertension or other blood pressure problems may result from abnormal NO production. It has now been proved that the vasodilator response dependent upon the endothelium is decreased in the animal models of hypertension, as well as in cases of atherosclerosis and coronary disease in man.

15   - Role of NO in the nervous system:

Produced under the influence of the stimulation of presynaptic NMDA-type receptors by glutamate, and acting by increasing the GMPc postsynaptic intraneuronal rate in both murines and man (Green et al., 1991), NO exerts a signaling action in the nervous
20   system which is both central (Bredt and Snyder, 1989) and peripheral.

In neurophysiology, NO is now considered to be a biological messenger (Garthwaite, 1991) or retrograde messenger (Barinaga, 1991; Feldman et al., 1993). It may play a long-term role in the potentiation (PLT) in the hippocampus. PLT is a phenomenon
25   corresponding to a specific increase in the efficacy of synaptic transmission favoring memorization. This indicates involvement of NO production in certain forms of learning which are dependent upon the hippocampus (Bredt and Snyder, 1990; Bredt et al., 1991; Zhuo et al., 1993).

30   NO is a non-cholinergic, non-adrenergic neurotransmitter and mediator of digestive tract relaxation (Desai et al., 1991), and participates just as well in neuronal control of cerebral vascular tone (Beckman, 1991) or erection (Rajfer et al., 1992), as in insulin secretion by the b cells in the islets of Langerhans (Schmidt et al., 1992).

Moreover, NO is involved in the process of brain destruction, after acute shock, such as ischemia-hypoxia (Moncada et al., 1991; Snyder and Bredt, 1992; Choi, 1993). The NO formed during the course of the ischemia becomes neurotoxic because of several mechanisms. The NO reacts with the superoxide ions to form peroxynitrites which initiate lipid peroxydation (Beckman, 1990). The NO is capable of blocking mitochondrial respiration (Drapier et al., 1988) and inhibiting the synthesis of DNA (Kwon et al., 1993). In addition to these direct cytotoxic effects, NO is thought to exert an effect promoting release of glutamate during ischemia (Buisson et al., 1993) which triggers the entry of calcium ions binding to calmodulin, thereby leading to NOS activation. The NO produced diffuses through the cell membranes and reaches the target cells, where it binds to the iron atom on the soluble cyclase guanylate active site. This in turn produces GMPc. In addition, released NO can act presynaptically to facilitate glutamate release.

However, it has been demonstrated *in vitro* that the direct addition of NO or NO-releasing molecules in neuronal cultures blocks activation of NMDA receptors (Manzoni et al., 1992). These observations have led the authors to suggest that endogenous NO synthesized by the neurons exerts retro-inhibition activity vis-à-vis the NMDA receptors. This retro-control is thought to be exerted by decreasing the frequency of opening of the channels linked to receptors and as a result reducing the influence of calcium. This effect is thought to be due to blockage by NO of the NMDA receptor modulating site (Lipton et al., 1993).

- Role of NO in the immune system:

The first studies of NO primarily demonstrated its toxicity. It had previously been thought that the nitrates excreted by man were of only alimentary origin. In 1981, Tannenbaum's group noted that the individuals and rats which consumed few alimentary nitrates still excreted large quantities of it. Food was not therefore the only source of nitrates. In addition, a subject with infectious diarrhea excreted a lot of urinary nitrates: formation of these nitrates seemed to be linked to the inflammatory process. More recently, a team noted that the injection of bacterial endotoxins stimulated the excretion of nitrates in the rat (Green et al., 1981; Snyder and Bredt, 1992).

In fact, NO enters into antitumor defense, in particular in the cytotoxic mechanisms mediated by macrophages (Stuehr and Marletta, 1985; Hibbs et al., 1987a, b; Drapier et al., 1988; Stuehr and Nathan, 1989; Keller et al., 1990).

5   The activation of murine peritoneal macrophages, whether *in vivo* with pathogens induced by cellular multiplication such as *Mycobaterium bovis,* or *in vitro* with cytokines, in particular gamma-interferon (IFN-γ), makes them cytotoxic to the target tumoral cells, and the cytotoxic mechanisms implemented are not involved in phagocytosis. It was then demonstrated that these activated macrophages express iNOS and synthesize
10   NO_3- (Stuehr and Marletta, 1987a) and NO_2- in response to stimulation by IFN-g, alone or in combination with lipopolysaccharide (LPS) or with alpha-tumor necrosis factor (TFN-α) (Stuehr and Marletta 1987a,b; Ding et al; 1988). Thus, the cytotoxicity of macrophages activated against tumor cells was established (Hibbs et al., 1987b) as depending upon the concentration of L-arginine. These authors also demonstrated that the activated
15   macrophages synthesize L-citrullin and NO_2- from arginine and that L-NMMA inhibits the synthesis of these two products and expression of cytotoxicity (Hibbs et al., 1987b).

The role of NO as a mediator of cytotoxicity was first demonstrated (Hibbs et al., 1988) and the metabolic pathway of L-arginine was considered to be an important
20   defense mechanism against intra- and extracellular microorganisms, parasites, bacteria and fungi (Hibbs et al., 1990).

NO acts on these targets in particular by binding to the iron-sulfur in proteins (Drapier et al., 1991; Feldman et al., 1993). It was also noted that a massive loss of
·25   intracellular iron is a possible cause of tumoral cell lysis induced by activated macrophages. NO seems to act on the active site of different enzymes acting on the replication of DNA (reductase ribonucleotides, RNR) (Lepoivre et al., 1990) and the citric acid cycle (aconitase) (Drapier and Hibbs, 1986) or in mitochondrial respiration (complexes I and II of the electron transport chain) (Granger and Lehninger, 1982; Drapier and Hibbs, 1988).
30   Electronic paramagnetic resonance studies (EPR) have demonstrated that NO disturbs the spatial configuration of iron-sulfur structures of certain enzymes, by forming iron-nitrosyl complexes (NO-Fe-SR), which has the effect of inhibiting enzymatic activity (Drapier and Hibbs, 1988; Lancaster, 1990; Pellat, 1990; Drapier, 1991). This phenomenon occurs in the presence of elevated quantities of NO, produced for several hours. It is noted that the

production of NO can also be induced in target cells themselves, for example, during tumoral cell autodestruction, or bacterial autotoxicity (Heiss et al., 1994).

In the mechanism of action of the anti-infectious effect, it has also been
5   demonstrated that NO can react with oxygen and produce different toxins such as the hydroxyl radical (OH°) or nitric dioxide (NO_2) (Stamler et al., 1992a). The peroxynitrite ion, one of the most oxidizing and cytotoxic of the NO derivatives, is now proposed as the principal mediator of the cytotoxic activity of NO. It is thought to intervene in the nitration of tyrosine radicals of certain cellular proteins (Beckman et al., 1994a). It is also known that
10   certain S-nitrosylated compounds (S-nitrosocysteine, S-nitrosoglutathione), and NO donor thionitrites can be strongly microbicidal, antiviral or anticancerous (Maul, 1993; Roy et al., 1995).

NO seems to have been recently identified in the rat and mouse as being one
15   of the mediators of immunosupression due to macrophages. An elevated concentration of L-arginine occurs during the acquisition of the suppressor characteristic (Albina, 1989a;b; Hoffman et al., 1990; Mills, 1991). Also, the addition of NMMA increases lymphocytic proliferation (Mills, 1991; Nussler et al., 1995). This immunomodulating role of NO was confirmed by other groups using Hb (Albina and Henry, 1991; Mills, 1991), or anti-IFNg
20   monoclonal antibody in spleen macrophage-cell cellular co-cultures in order to induce lymphocytic proliferation (Albina et al., 1991). NO is thought to preferentially inhibit the proliferation of Th1 lymphocytes, and to exercise retrocontrol in its production.

In certain trypanosomoses, in which immunosuppression is very marked,
25   macrophages exert a strong suppressive activity with mechanisms involving prostaglandins and NO (Schleifer, and Mansfield, 1993).

- NO and Hemoproteins:
NO binds to numerous hemoproteins (Fe II) *in vitro*. It sometimes also binds
30   to their ferric form (Henry et al., 1991). Moreover, when inhaled, NO is a harmful gas because it binds to the ferrous iron in hemoproteins (Boucher et al., 1993; Rossaint et al., 1993). The best known example is hemoglobin. NO is capable of rapidly binding to desoxyhemoglobin (Hb[FeII] to form the Hb complex Hb[FeII]NO which is relatively stable (t1/2 = 12 min). In fact, NO's affinity for desoxyhemoglobin is 106 times greater

than that of oxygen. Oxyhemoglobin (Hb[FeII])O_2) can be converted by NO to methemoglobin (t1/2 = 20h) and nitrate (Ducrocq et al., 1994).

In addition, hemoglobin may play a role as NO transporter or oxidizer
5   (Henry et al., 1991; Traylor and Sharma, 1992; Boucher et al., 1993). In fact, in man, each hemoglobin sub-unit contains a heme group, and the b sub-units contain very reactive thiol radicals (Cys b 93), forming S-nitrosohemoglobin. This nitrosylated form of hemoglobin was recently identified as not only a regulating factor for vascular, pulmonary and systemic activity, but also as an element of transnitrosylation (Jia et al., 1996).
10

Other hemoproteins are activated by NO, such as guanylate cyclase in which the NO receptor is the iron atom. NO binding deforms the heme group (which contains the iron atom), and activates the production of the second messenger GMPc from GTP, inducing vasodilation and inhibition of platelet aggregation (Ignarro, 1991; Moncada et al.,
15   1991; Schrrtidt, 1992; Snyder and Bredt, 1992). Also included are prostaglandin H synthase (Salvemini et al., 1993; Stadler et al., 1993) and others which are more or less reversibly inhibited (Rogers and Ignarro, 1992; Assreuy et al., 1993; Rengasamy and Johns, 1993) such as P-450 cytochromes (Adams et al., 1992; Ducrocq et al., 1994) and the NOS themselves (Rogers and Ignarro, 1992; Assreuy et al., 1993; Rengasamy and Johns, 1993).
20

In conclusion, NO appears in different transport forms; the enzymatic mechanisms of synthesis, storage and transport are performed as a function of its role as intermediary and effector (Stamler et al., 1992a, 1994).

25   - NO and cancerogenesis:

In different situations, NO seems to play several roles, which are sometimes even antagonistic. This is true in cancerogenesis (Calmels and Ohshima, 1995). In contrast to its (bactericide and cytotoxic) role in bodily defenses, NO is capable of causing DNA
30   changes and thus promoting the development of cancer. This phenomenon has, however, most often been demonstrated in *in vitro* work frequently not related to actual physiological conditions.

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Several distinct roles of NO in the various stages of cancerogenesis have been noted:

(i) formation of peroxynitrite, a naturally cytotoxic product, which after protonation, can also break down into $OH°$ and $NO_2°$ (Beckman et al., 1990). These radicals are responsible in part for the oxidative damage to the DNA permitting the later neoplastic transformation of the affected cells;

(ii) genotoxicity of NO and deamination of DNA: NO can induce mutations in human lymphoblastoid cell lines (Nguyen et al., 1992);

(iii) formation of nitrosamines: NO following oxidation is capable of reacting with a nitrosable substrate to form nitrosamines. These are powerful carcinogens in animals and are also thought to be the cause of certain cancers in man (Loser et al.; 1989; Jacob and Belleville, 1991).

NO can also promote the development of cancer through immunosupression (Hoffman et al., 1990; Albina et al., 1991; Mills, 1991). Studies have demonstrated, for example, that its presence seems to promote the process of neoplastic transformation of *in vitro* murine fibroblasts (Werner-Felmayer et al., 1993). However, recent work indicates a somewhat controversial protective effect of NO vis-à-vis cytotoxicity induced by oxygenated species (Winck et al., 1993) and vis-à-vis the progression of skin tumors induced under W in the mouse (Yuin et al., 1993; Calmels and Ohshima, 1995). Finally, despite its role in cancerogenesis, NO is also an important effector element vis-à-vis tumor cells (Hibbs et al., 1990). In addition, NO can also mediate the apoptosis of different cell types (Sarih et al., 1993; Me, Bmer et al., 1994; Fehsel et al., 1995). All these data demonstrate the various influences of NO.

- NO and autoimmune inflammatory diseases:

It has recently been discovered that NO is involved in certain inflammatory pathologies (Schmidt and Walter, 1994; Moilanen and Vapaatalo, 1995). Multiple sclerosis (MS) (Sherman et al., 1992) and rheumatoid arthritis (RA) (Grabowski et al., 1996) are the diseases that have been studied most extensively.

22

The Inventors studied these two pathologies in order to demonstrate the effect of NO and its nitrated or nitrosylated derivatives in their clinical development. The studies are based on experimental models of multiple sclerosis (experimental autoimmune

5   encephalitis) and RA (experimental inflammatory arthritis).

The mechanisms of action of NO, whether direct or through derivatives, are still poorly understood. Because of its nature, NO can diffuse through the membranes of cells neighboring those where it was synthesized. This NO diffusion may explain a local

10   effect at a very short distance due to its strong reactivity. It is probably this mechanism which causes activation of the guanylate cyclase of the smooth muscle cells of the vessels by NO synthesized by the endothelial cells. However, long-distance action of the NO may also be possible. This long-distance action may be linked to the existence of molecules capable of binding the NO and then releasing it. Among the compounds capable of playing

15   this role, S-nitrosothiol compounds play a significant role. Likewise, since 1981, S-nitrosothiols have been implicated in vasodilation phenomena (Ignarro et al.).

More recently, nitrosothiols have also been demonstrated in the cells of the immune system (Dias-Da-Motta et al., 1996; Wagner et al., 1996; Zhao et al., 1996). The

20   mechanisms of NO release by these molecules are poorly understood, in particular the reactions leading to passage of NO on other molecules. In addition, is it always the $NO°$ form or can the NO^+ forms be involved in nitrosylation mechanisms? Can other radicals, in particular O_2 derivatives, also be involved? Whether the methods of action of NO are local or long-distance, there may be intermediate molecules with greater properties and stability

25   than NO whose function is not yet known.

The antibodies of the invention first permitted studying the stability and properties of nitrosylated compounds, and they were then used to mask the nitrosylated epitopes appearing in certain pathological states in which excess production of NO has

30   been verified. Elevated production of these nitrosylated derivatives may be responsible, at least in part, for the problems observed.

II - <u>PREPARATION OF POLYCLONAL ANTIBODIES DIRECTED AGAINST NITROSYLATED CONJUGATES.</u>

1) <u>Immune response.</u>

Destruction of infectious agents is produced by a group of elements belonging to the immune system. This protection is obtained from a humoral and/or cellular immune response whose main players are lymphocytic and macrophagic cells (Oberg et al., 1993). B lymphocytes favor the humoral response via production of antibodies specifically recognizing a given antigen, sometimes accompanying the neutralization of harmful effect of this given antigen. T helper lymphocytes (Th1) intervene in the activation of macrophages by secreting cytokines such as IFN-γ and TFN-α/β. Activated macrophages release different molecules termed effectors which exert a cytostatic or cytotoxic effect vis-à-vis intra- or extracellular microorganisms. These molecules include free radicals, in particular NO (Liew, 1991; Ahvazi et al., 1995; Blasi et al., 1995; Juretic et al., 1995; Lin et al. 1995) which, by binding to molecular targets, generate *in vivo* nitrosylated neoantigens which can induce a humoral response.

In order to reproduce the structure of these neoantigens, the Inventors synthesized artificial conjugates by coupling NO with two amino acids, tyrosine and cystein. With these conjugates, they induced polyclonal antibodies directed against NO-Tyr and NO-Cys epitopes in the rabbit.

2) <u>Immunochemistry of haptens.</u>

The immunochemistry of haptens, or small molecules, is based on specific recognition of a particular antigen by an antibody (Ab) in a highly selective way (Landsteiner, 1945; Kabat, 1968; Goodman, 1975). Haptens such as amino acids (cysteine, tyrosine, and tryptophan.) have a low molecular weight (75-300 daltons) and are not, therefore, immunogenic by themselves. The development of specific antibodies is based on a certain number of methodological principles and concepts. Thus:

- A substance is immunogenic if it is capable of stimulating antibody production; it must be larger than 1000 daltons.

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- A compound is antigenic if it enters into a specific interaction with an antibody site. To do so, it must be able to distinguish structurally similar conjugates, which differ by the position of a chemical group on the hapten (Landsteiner, 1945; Geffard et al., 1985a);

5

- The antigen-antibody bond is established through different types of bonding: hydrogen, Van Der Walls, ionic, hydrophobic.

For this approach, the Inventors first synthesized nitrosylated conjugates,

10   then conjugates which were structurally analogous to them. The study focused exclusively on nitrosylated conjugates and their non-nitrolysated corresponding conjugates such as nitrated conjugates. With these tools, nitrosylated anti-hapten polyclonal antibodies coupled with carrier proteins were produced and characterized using carbodiimide as a coupling agent.

15

3) Materials and methods.
a) Synthesis of NO-tyrosine and NO-cysteine immunogens coupled with a carrier protein by a "carbodiimide" type of coupling:

20   Immunogens are synthesized by first coupling the L-tyrosine (Tyr) or L-cysteine-N-acetylated (Cys) hapten to a carrier protein such as bovine serum albumin (BSA) or human serum albumin (HSA) by carbodiimide according to the procedure indicated below (Harlow and Lane, 1988). The conjugate obtained were then nitrolysated by an NO donor.

25

- 10 mg of Tyr (Fluka), or 20 mg of Cys (Sigma) are solubilized in the MES (2[N-Morpholino] ethanesulfonic acid, Sigma) (0.2 M) pH 5.4 buffer, and then a microliter of radioactive isotope [^{3}H-Tyr] (NEN, specific activity: 31.0 ci/mmole) is added. 20 mg of BSA or HSA dissolved in the same MES buffer is then added to conjugate the hapten with

30   an amide bond. Activation of the carboxyl grouping of the Tyr or Cys is initiated by the addition of carbodiimide [1-ethyl-3 (3-dimethylamino propyl) carbodiimide, Sigma]. The non-bound Tyr and Cys molecules are eliminated by dialysis with distilled water for 24 hours at 4° C.

The radioactive label enables determination of the conjugated hapten concentration as follows:

Concentration (M) in coupled hapten = X mg hap x CPM after / CPM before

5   x Vol before x PM hap

where X mg is the quantity of hapten used for the coupling; CPM av is the radioactivity before dialysis; CPM after is the radioactivity after dialysis; Vol before is the volume before dialysis; PM hap is the molecular weight of the hapten.

10   - In order to calculate the protein concentration in accordance with Beer-Lambert's law OD = e c l (in this work the length of cell l = 1 cm), absorbency with wavelengths of 280 nm et 300 nm was used (Geffard et al., 1985a):

Concentration (M) in protein = OD 280 nm - OD 300 nm / e protein

where e is the molar extinction coefficient of the protein at 280 nm; it is

15   34,500 for BSA and HSA;

For haptens which absorb at 280 nm (Tyrosine, Tryptophane), the Inventors used the following formula to determine the protein concentration :

$D°280 = C_{hap} \times e_{hap} + C_{protein} \times e_{protein}$

- The coupling relationship is the number of moles of hapten coupled

20   with a mole of protein:

Relationship (R) = concentration in hapten / concentration in protein.

- The conjugated weight is determined using the following relationship:

The conjugated Weight = [(R x PMhap) + PM prot] x conc protein.

Where the concentration (conc) is expressed in M. The weight is in

25   mg/ml.

- Nitrosylation of neosynthetized conjugates :

Nitrosylation takes place using a chemical NO donor, sodium nitrite ($NaNO_2$) in an acid medium (Stamler et al., 1992d). Thus, the formation of the

30   NO-hapten bond was evaluated by spectrophotometry measuring the OD between 320 and 500 nm. In addition, S-nitrosylation was proved by the (1958) method using mercury chloride $HgCl_2$. In fact, $HgCl_2$ in the presence of the S-nitrosylated compound leads to immediate release of the nitrogen bound to the sulfur of the nitrosothiol radical. This rapid hydrolysis may be explained by the affinity of Hg^{2+} ions for sulfur.

Table I below demonstrates the structure of two nitrosylated haptens bound to the carrier protein BSA.

Table 1

Conjugates	**Molecular Structures**
Tyrosine –BSA	HO—⟨⟩—CH_2-CH-CO-NH-BSA, with NH₂ branch: CH_2-$\overset{\displaystyle \mid}{CH}$-CO-NH-BSA, NH_2
NO-Tyrosine-BSA	HO—⟨⟩—CH_2-CH-CO-NH-BSA, NO substituent on ring, NH_2 branch
Acetylated- Cystein-BSA	SH-CH_2-$\overset{\displaystyle \mid}{CH}$-CO-NH-BSA, NH-CO-$CH_3$
NO-Acetylated-Cystein-BSA	NO-S-CH_2-$\overset{\displaystyle \mid}{CH}$-CO-NH-BSA, NH-CO-$CH_3$

b) Synthesis of other nitrosylated and nitrated conjugates :

The use of different coupling agents such as carbodiimide, glutaralgehyde (G) or succinic anhydride (SA), yields epitopes with a distinct conformation.

- "Carbodiimide" type:

Synthesis of NO-Tryptophan-BSA : Carbodiimide, tryptophan (Tryp, Sigma) and BSA were used for the synthesis of the Trp-BSA conjugate. This conjugate was then nitrosylated using $NaNO_2$.

- "Glutaraldehyde" type:

Synthesis of NO-Tyr-G-BSA, NO-Cys-G-BSA, and NO-Tryp-G-BSA: 5 mg of each hapten: Tyr, Cys (non acetylated) or Tryp were dissolved in an acetate buffer (1.5 M), pH 8.3. To follow the coupling reaction, a small quantity of tritiated hapten was added. Then an aqueous solution of glutaraldehyde (G) 5% (Merck) was mixed into the hapten solution. After several seconds, the protein (10 mg) diluted in the same buffer was mixed with the hapten solution until a yellowish color appeared. The reaction was then halted by adding 200 µl of a $NaBH_4$ (10 mM) solution; when the mixture became translucid, each solution of the different conjugates was set aside to dialyze with distilled water for 24 hours at 4°C (Atassi, 1984).

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- "Succinic Anhydride type" :

- Syntheses of NO-Tyr-SA-BSA and NO-Cys-SA-BSA : 20 mg of Tyr or Cys (non acetylated) were taken up in 200 µl of Diméthyl-sulfoxide (DMSO, Merck) and 800 µl of distilled water. Succinylation was induced by the addition of 17 mg of succinic anhydride (SA) (Sigma), and a quantity of NaOH 1N was added to neutralize the mixture, which was then freeze-dried.

5 mg of succinylated hapten were dissolved in 1 ml of pure, anhydrous dimethylformamide (DMF, Merck), containing 50 µl of pure, anhydrous triethylamine (TEA, Merck). The free COOH groups of the succinylated hapten are activated by adding 200 µl of Ethyl chloroformate (ECF, Prolabo) prediluted to 1/6. Then, the solution containing 10 mg of BSA is added. The reaction mixture is dialyzed in distilled water for 24 hours at 4°C under agitation.

The nitrosylation method of two types of coupling (G and SA) is identical to the one described above for the carbodiimide type of coupling.

- Synthesis of NO_2-tyrosine-BSA : The synthesis of this conjugate requires 20 mg of the NO_2-Tyr (Sigma) hapten and 20 mg of BSA. The coupling takes place with carbodiimide following the same protocol described above.

Synthesis of NO-protein conjugates : This form of nitrosylated BSA or S-nitroso-BSA was prepared from the BSA and sodium nitrite. The same quantity of protein and $NaNO_2$ was weighed for the NO-BSA coupling using the Stamler method (1992d).

c) Obtaining and characterization of anti-NO conjugated polyclonal Ab :
- Immunization of rabbits :

Starting with the NO-Tyr and NO-Cys conjugates, the Inventors immunized rabbits subcutaneously (Vaitukaitis et al., 1971): le "T" rabbit with the NO-Tyr-protein (BSA or HSA) conjugates, and the "C" rabbit" with the NO-Cys-protein conjugate. The rabbits were immunized by alternating these two carrier proteins (Geffard et al., 1984a, b; 1985a). The immunization was done by injecting an emulsion containing 200 µg of the conjugate, Freund's complete adjuvant (ACF, DIFCO) and a saline phosphate buffer (SPB). Antiserum sampling was done between 12-21 days after each injection.

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- Purification of immunoglobulins :

An immune response was induced. To enrich the serums with immunoglobulins (Ig) specifically directed against the coupled haptens, the following purification methods were used:

- Adsorption of polyclonal serums on carrier proteins :

The polyclonal serums were adsorbed on the corresponding non-nitrolysated conjugates: Tyr-BSA/HSA for the "T" rabbit and Cys-BSA/HSA for the "C" rabbit. (Geffard et al., 1984a; Geffard et al., 1985b; Campistron et al., 1986). The adsorption took place in proportions of 5 mg of conjugate per ml of pure serum. The mixture was incubated for 16 hours at 4°C under agitation and the immunoprecipitates were eliminated by centrifugation for 15 minutes at 10000g. The supernatant is enriched in specific Ig, while the pellet contains the rabbit Ig-carrier protein immune complexes.

- Purification of Ig by precipitation in ammonium sulfate :

To one volume of rabbit polyclonal serum an equal volume of a saturated ammonium sulfate solution (NH4)2SO4 is added. The mixture was incubated for 1 hour at 4°C, and then centrifuged for 15 minutes at 10 OOOg. The cell (containing the Ig precipitates) is taken up in a minimum volume of TPB buffer and then dialyzed for 3 days in a SPB buffer (Na2HPO4, 0.01 M, NaCl 0.15 M).

- Purification of Ig with exclusion chromatography :

After precipitation with ammonium sulfate, the Ig were separated by molecular weight with the technique of exclusion chromatography in a G 200 (Séphadex) column (Mons and Geffard, 1987). Determination of the concentration en Ig was deduced from the spectrophotometric analysis of one diluted aliquot, and it was then estimated as follows:

1.4 unit of OD at 280 nm corresponds to one quantity of 1 mg/ml of Ig.

The specific Ig are primarily Ig G. The supernatants purified according to one of these methods were used to study title, avidity and specificity using the ELISA test.

d) Immunoenzymatic test (ELISA method) :

The immunoenzymatic test is comprised of four primary steps (Engvall et al., 1972):

1st step : Covering of the microtitration plates. This step involves adsorption of conjugates: NO-Tyr-BSA and Tyr BSA for the "T" rabbit or NO-Cys-BSA and Cys-BSA for the "C" rabbit, in wells of microtitration plates (certified Maxisorp, NUNC). This covering phase was also done with the coupled "G" conjugates", "SA" and for BSA and NO-BSA.

2nd step : Saturation. It is necessary to "saturate" the well to avoid non-specific attachment. This stage is done with the following buffer: SPB-tween-glycérol-BSA. Tween 0.05 % pH = 7.2, glycerol 10%. BSA concentration was optimized to 5 g/l.

3rd step : Formation of primary complexes. This involves fixation of primary Ab complexes potentially contained in the serum to be tested at an optimal dilution of 1/20,000 (for the "T" et "C" rabbits) on the conjugates placed in the wells.

4th step : Formation of secondary complexes. This involves the addition of anti-Ig rabbit secondary goat Ab (Pasteur Diagnostic) labeled with peroxidase diluted to 1/8000 in the SPB-Tween-BSA buffer.

5th step : Visualization of secondary complexes is done using the specific peroxidase substrate (H_2O_2, Prolabo), in the presence of a chromogen, orthophenyl diamine (OPD, Sigma). It is visualized with oxidation of the OPD which changes from a colorless reduced state to a yellow oxidized state. The reaction is then stopped by adding a sulfuric acid solution (H_2SO_4, 4N) in each well. The OD were read at 492 nm using a computerized multiscan spectrophotometer (EL 312e, Bio-Tek Instruments). The experimental value obtained was measured in the wells with the conjugate (hapten-nitrosylated-protein) or nitrosylated protein extracted from the one read in the control wells containing the non- nitrosylated conjugate or the BSA.

- Immunochemical characterization of the Ab sites :

This characterization is based on two Ab properties: avidity and specificity.

The avidity of each antiserum was evaluated as follows : Dilutions of NO-Tyr-BSA conjugates for the anti NO-Tyr antiserum or NO-Cys-BSA for the antiserum anti NO-Cys, from 2 x 10^{-5} M to 2x10^{-12} M in SPB-Tween-BSA glycerol were prepared. This competition took place by the incubation of Antiserum ("T" or "C") in the presence of the immunogen for one night at 4°C. The test then took place as described above using these dilutions as primary Ab.

30

For specificity: the compounds used for the competition experiments were as follows for the two types of Ac: NO-Tyr-BSA; Tyr-BSA; NO-Cys-BSA; Cys-BSA; NO-Cys(non acetylated)-BSA; Cys(non acetylated)-BSA; NO-Tyr-G-BSA; Tyr-G-BSA; NO-Cys-G-BSA; Cys-G-BSA; NO-Tyr-SA-BSA; Tyr-SABSA; NO-Cys-SA-BSA; Cys-SA-BSA; NO2-Tyr-BSA; NO-Tryp-BSA; Tryp-BSA; NO-Tryp-G-BSA; Tryp-G-BSA; NO-BSA.

At the end of the competition, the displacement curves for the different compounds tested were established.

4) Results.
a) Analysis of nitrosylated conjugates :
- Spectrophotometric analysis of conjugates: A spectrophotometric test was used to follow the different stages of immunogen synthesis. This occurred between the wavelengths of 240 and 500 nm. To compare the spectrums obtained before and after nitrosylation, the Inventors determined the absorbency zone of bound NO. As demonstrated respectively on the curves of figures 1 and 2, the zone of absorbency of NO-Tyr-BSA was noted to be between 320 et 440 nm (figure 1) et that of NO-Cys-BSA was noted between 320 and 400 nm (figure 2). An absence of absorbency between the wavelengths of 320 et 500 nm was noted in the spectrums of conjugates: Tyr-BSA et le Cys-BSA;

The absorbency band of the same NO-Cys-BSA conjugate was not found after the conjugate was treated with a solution of lmM of HgCl2. This demonstrates that the covalent bond "S-NO" had been abolished.

b) Immunochemical analysis of Ab sites.
- Evaluation of Ab titer directed against the epitopes NO-Tyr and NO-Cys:

For the "T" rabbit immunized with the conjugated NO-Tyr, the specific Ab titres evolved after each immunization and the optimal response was obtained after the third immunization. Therefore, as demonstrated by the curve of figure 3, two types of responses were visualized for the "T" rabbits. The first is directed against the NO-Tyr-BSA conjugate which remained more significant during the immunization than the second directed against the Tyr-BSA conjugate. In Figure 3, the white arrow

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indicates the sample before immunization and the black arrows indicate the sampling days after each immunization.

- For the "C" rabbit immunized by the conjugated NO-Cys, no specific response was obtained before the 5th immunization. Before this immunization, the Inventors observed a more significant anti-Cys-BSA response relative to the anti NO-Cys-BSA. However, following the 5th immunization the Inventors detected a decline in the non-specific response. Figure 4 demonstrates the evolution of two types of response in the rabbit : one corresponding to a global response in the NO-Cys-BSA conjugate including the antibodies directed against the carrier protein and a second directed against the Cys-BSA conjugate. In figure 4, the white arrow indicates the sample before immunization and the black arrows indicate the sampling days after each immunization.

The sampling of the "T" rabbit after the 3rd immunization was used for the immunochemical characterization of the Ab specifically directed against the nitrosylated epitope. Likewise, the Inventors used a sample after the 5th immunization for rabbit "C".

- Determination of the avidity and specificity of developed Ab :

The avidity of conjugated anti-NO-Tyr Ab and conjugated anti-NO-Cys was evaluated by competition tests. Inhibition of the binding of "T" and "C" anti-serums was obtained by incubating each of these two Ab with the nitrosylated conjugate which served as an immunogen.

Decrease in the OD (B) indicates the presence of competition between the conjugated hapten which is adsorbed on the microtitration plate and the hapten preincubated with the corresponding antiserum. Bo is the OD corresponding to the response obtained with the antiserum in the absence of the competitor. A dilution of the antiserum (1/20,000) yielding a OD of approximately 1.0 to 492 nm was chosen for adjustment of the value of Bo; the B/Bo relationship was used to trace the competition curves of figure 5 obtained with the competitors.

- For the "T" rabbit, the avidity determined at the half-placement was 1.1 x 10^{-8} M (figure 1, curve 1) and for the "C" rabbit", the avidity was 4.5×10^{-8} M.

The specificity of each antiserum was also evaluated by competition experiments between the Tyr-BSA conjugate (for the "T" antiserum), or NO-Cys-BSA (for the "C" antiserum) and the conjugated structural analogues. The displacement

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curves were obtained from the results of competition tests with the conjugated haptens which were not used during the immunization. By using the B/Bo relationship for each of the competitors, the Inventors were able to determine the corresponding specificities :

- For the "T" rabbit" the best recognized conjugate was the immunogen which yielded an avidity of 1.1×10^{-8} M. The other conjugates recognized were: NO2-Tyr-BSA (ICso = 1.4×10^{-7} M); NO-Tyr-SA-BSA (ICso = 5×10^{-5} M); and NO-Tryp-G-BSA (ICso = 3×10^{-6} M). Curve 2 of figure 5 also demonstrates the absence of recognition of Tyr-BSA by this antiserum.

- For the "C" rabbit the immunogen NO-Cys-BSA and the conjugate NO-Cys(non acetylated)-BSA were recognized with an avidity of 4.5×10^{-8} M and a specificity of $\sim 10^{-6}$ M.

All the other conjugates, whether nitrosylated or not, (NO-BSA for example) were not recognized by the "T" Ab or the "C" Ab.

(IC_{50} is the molar concentration of the competitor yielding 50 % inhibition).

It should also be noted that indirect ELISA tests demonstrated discrimination between the NO-BSA and BSA by the "T" and "C" polyclonal Ab. The OD obtained with these antiserums are respectively on the order of 0.34 ± 0.062 and 0.27 ± 0.05. However, in liquid phase, the NO-BSA was not recognized which may be explained by the conformational modification of this molecule in solid phase (indirect test) and liquid phase (competition test).

5) Discussion.

The development of the anti-NO conjugate Ab was created, based on the work of Landsteiner, (1945); Goodman, (1975); Geffard et al., (1985b); and Mons and Geffard, (1987) for induction of antiserums against small molecules. The reactivity of NO with the thiol groups may explain, in part, its role in different biological processes (Girard and Potier, 1993). It has in fact, been postulated that NO is stabilized in the form of S-nitrosothiol which may retain its biological reactivity. The present immunological study is based on the hypothesis of the stability of NO derivatives. The Inventors used different nitrosylated conjugates thought to be NO transporters (nitrosylated aryl radicals and S-nitroso-Cys). The immunochemical characterization of anti-NO-Tyr and developed anti-NO-Cys demonstrates the different power of discrimination of these Ab for the corresponding immunogens. In addition, the specific optimal nitrosylated

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anti-Tyr titer was noted after the 3rd immunization, at the end of 1-2 months (figure 3), whereas the nitrosylated anti-Cys does not demonstrate evolution in the titer before a period of 2-3 months (figure 4), et yields an optimal titer after the 5th immunization.

Relative to the specificity of these preincubated Ab with other conjugates

5   that are structurally analogous with the compound used as an immunogen, a difference in the responses obtained with these two Ab is also noted. Thus, the "T" Ab recognizes two conjugates which were not used in the immunization, N0$_2$-Tyr-BSA and lNO-Tryp-BSA, which is not the case of the "C" Ab. None of the other non-nitrosylated conjugates was recognized by these two Ab. The different responses noted with these

10 two types of Ab may also be explained by the differences in the molecular structure of the immunogens, their stability in a biological medium, their metabolism, or by the modifications favored by the cells presenting the antigen. All these factors may play a fundamental role in stimulation of the immune system and the avidity of the induced Ab.

Finally, recognition of the NO-BSA chemically synthesized by the

15 polyclonal Antibodies led the Inventors to use this form of NO transport in multiple *in vitro* applications, utilizing activated macrophages as biological carriers of NO.

III - DETECTION AND NEUTRALIZATION OF NO DERIVATIVES BY POLYCLONAL ANTIBODIES.

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The macrophage cells play an essential role in the immune response. In fact, as soon as an infection develops, they may intervene to destroy the microorganism through phagocytization. In addition, the macrophages activated during a specific immune response exert a cytotoxic microbicide effect on intracellular microorganisms

25 (Ruskin and Remington, 1968). One of the metabolic pathways of arginine is essential in activation of the macrophages and their cytotoxic effect on tumor cells (Hibbs et al., 1987a) and cytostatic effect on fungal species such as *Cryptosoccus neoformans* (Granger et al., 1988). This metabolic pathway leads to the synthesis of L-citrulline and NO from arginine (Hibbs et al., 1987b; Hibbs et al., 1988), due to iNOS. The structural

30 analogue of arginine, NMMA was then characterized as a competitive inhibitor of this synthesis from activated macrophages (Hibbs et al., 1987a, b; Drapier and Hibbs 1988; Hibbs et al., 1988).

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This cytotoxic phenomenon was primarily studied *in vitro* in the murine macrophages and there is now a considerable degree of consensus in affirming that it is based on the L-arginine -> NO biosynthesis pathway. (Drapier, 1993).

The cytotoxic role of NO has also been interpreted as resulting from the formation of iron-nitrosyl compounds in the enzymes of oxidative metabolism of the target cells (Stuehr and Nathan 1989; Stuehr et al., 1989).

In conclusion, an infection induces stimulation of T lymphocytes via the presentation of the antigen. The T cells secrete IFN-γ in response to their activation and successive stimulation is maintained with the ongoing presence of the antigen or of reinfection. The IFN-γ then activates the macrophages which produce other cytokines such as TNF-α and IL-1 which, in synergy with the IFN-γ can stimulate the macrophages. The macrophagic stimulation permits the elimination of certain tumor cells and numerous pathogenic agents. Among the different mechanisms developed by the macrophages, one of the most effective is the synthesis of NO. The destruction of leishmaniasis and schistosoma can be completely inhibited by NMMA (James and Glaven, 1989; Green et al., 1990; Liew et al., 1990). This seems to indicate that in these cases NO is necessary, and may even be sufficient, to explain the microbicide role of macrophages. NO therefore plays a vital role in defense of the body.

The purpose of the work reported below is to demonstrate the form in which NO is transported to act against its targets. In order to simulate physiological conditions, the Inventors used a biological source of NO (macrophages activated by the BCG), BSA as NO transporter and extracellular trypanosomes. The goal was to detect nitrosylated epitopes formed *in vitro* with a cytotoxic role and to block them by using our polyclonal conjugated anti-NO-Tyr and anti-NO-Cys antibodies.

1) Material and methods.

a) Sampling and culture of macrophages :

Peritoneal macrophages from the Swiss mouse previously infected with BCG (Institut Pasteur) or normal macrophages, were cultured at 1.5×10^6 cells/ml/Petri dish, for 8 hours at 37°C in an incubator containing 5% CO2. The culture medium used was HBSS (Hank's Balanced Salt Solution, GIBCO). The culture was done in the presence or absence of NMMA 0.5 mM.

b) <u>Detection of the formation of NO and NO-BSA conjugates with the ELISA test</u> :

- <u>Competition tests between the nitrosylated conjugate formed in the culture medium and the NO-Tyr-BSA or NO-Cys-BSA neosynthetized conjugates:</u>

The supernatants from the culture mediums of the activated macrophages containing the nitrosylated conjugates (NO-Tyr-BSA or NO-Cys-BSA), were incubated in the presence of the "T" or "C" antiserum used at 1/20,000. These nitrosylated conjugates were formed from the NO produced by the macrophages and the Tyr-BSA or Cys-BSA conjugates added to the medium. After incubation these supernatants then served as primary Ab for the ELISA test. This test was performed on microtitration plates adsorbed by the NO-Tyr-BSA or NO-Cys-BSA conjugates. The rest of the test is identical to the one previously described.

The same protocol was used for the supernatant from the normal macrophage culture mediums (control). For this research, 5 experiments were done and good reproducibility was obtained with anti-NO-Cys.

- <u>Detection of nitrosylation of the epitopes carried by BSA using anti-NO-Tyr and anti-NO-Cys Ac:</u>

This application necessitated the use of delipidated BSA (4 mg/ml), to prevent lipoperoxidation ($NO + O_2^{-\circ}$) of fatty acids adsorbed onto the BSA. This lipoperoxidation yields compounds causing non-specific reactions. The protein was added to the culture medium (HBSS) of activated or non-activated macrophages for 8 hours. The microtitration plates were then adsorbed for 6 hours by the supernatants from the activated macrophage culture mediums (containing NO-BSA), non-activated macrophages or from the culture medium itself. After saturation the "T" or "C" primary Ab were used at a dilution of 1/1000.

- <u>Neutralization of the NO-BSA trypanostatic effect:</u>

The Partinico II strain of *T. musculi* was used. The trypanosomes were purified by passing them through a column of diethyl aminoethyl cellulose (Lanham, 1968). The trypanosomes were then co-cultivated for 8 hours in the presence of macrophages from normal mice or those infested with BCG in a RPMI medium containing the delipidated BSA (4 mg/ml). The polyclonal Ab (1/100) or NMMA (0.5 mM) were added in several wells to enable comparison of their inhibitor effects. Normal

rabbit serum and an anti-BSA polyclonal Ab were used as a control at the same dilution as the "T" and "C" antiserums.

- Neutralization of the transfer of this trypanostatic effect :

The purpose of this work was to confirm the trypanostatic effect of NO--BSA. To do this, the culture supernatant containing the BSA and macrophages activated by the BCG in the presence or absence of NMMA, was transferred after 8 hours of incubation to a co-culture of normal /T. musculi macrophages under the same conditions. The "T" "C" polyclonal Ac, normal rabbit serum or anti BSA diluted to 1/100 were applied with the supernatant.

Using these two methods, the Inventors first studied the trypanostatic cytostatic effect of the NO -BSA either directly or transferred. Then they compared the neutralization of this effect by the polyclonal Ab according to two slightly different procedures. Inhibition of the trypanostatic effect by the Ab under these two conditions proves, first, the synthesis of NO and therefore the formation of the NO-BSA and, second, that it is an effect which is à priori specific to NO-BSA and not to other oxygenated derivatives.

- Measurement of the quantity of nitrite produced :

The production of NO is represented by the formation of NO_2^- accumulated in the culture medium using a colorimetric reaction: the method of Griess (Hageman and Reed, 1980; Lepoivre et al., 1989). The light-sensitive reactant of Griess is obtained by mixing, volume for volume, sulfanilamide (1% in HCl 1.2 N; Sigma) and N-(1-naphtyl) ethylenediamine dihydrochlorate (3% in water; Sigma); a volume of 200 µl of reagent is added to 100 µl sample, incubated for 30 minutes in the dark, and then the absorbency at 550 nm is measured using a plate reader. A color chart is made for each dose using a solution of $NaNO_2$.

2) Results.

a) Detection of the formation of NO-Cys-BSA conjugate in a macrophage culture medium :

The NO produced in vitro by the macrophages activated by the BCG, induces the formation of NO-Cys-BSA from the Cys-BSA conjugate previously added to the medium. The latter plays the role of a target, a reservoir and stable form of NO produced. The nitrosylated derivative formed is detectable with competition

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experiments. In the presence of NMMA, inhibition of the synthesis of NO and the absence of signal was noted.

Figure 6 represents the kinetics of the formation and concentration in NO-Cys-BSA formed in the supernatant of the culture of activated macrophages determined at different incubation times: 0, 3, 4, 6, 8, 11, 14, 18, and 20 hours using the "C" antiserum. The same procedure was applied to non-activated cells and the culture medium itself with and without NMMA. The concentration (μM) of NO-Cys-BSA formed from the avidity curve of the "C" antiserum was then calculated.

The supernatants from the normal macrophage culture were tested in the same way, and no formation of nitrosylated conjugates was found.

b) Detection of nitrosylated conjugates with the ELISA test :

The nitrosylated BSA was detected with the ELISA test using the two "T" and "C" Ab diluted to 1/1000. These tests demonstrate recognition by these Ab of epitopes such as NO-Tyr and NO-Cys formed in the NO-BSA.

The two Ab yielded values of the same order with normal and activated macrophages, both with and without NMMA. In table 2 below the values represent the average and the standard-deviation of 5 experiments.

Table 2

	DO (492 nm)/NO-BSA			Concentration of NO_2^- (μM)
	NMMA 0.5mM	anti-NO-Tyr 1/1000	anti-NO-Cys 1/1000	
Macrophages	-	0.241 ± 0.051	0.261 ± 0.034	26.00 ± 2.00
BCG	+	0.096 ± 0.018	0.099 ± 0.034	3.00 ± 0.005
Macrophages	-	0.125 ± 0.031	0.116 ± 0.02	2.00 ± 1.00
Normal	+	0.143 ± 0.004	0.112 ± 0.018	2.00 ± 1.00

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In the presence of NMMA, the OD obtained with the activated macrophages is decreased by approximately 2.5 relative to those obtained in the absence of NMMA. However, with normal macrophages, a big difference in the OD obtained with or without NMMA is not noted, leading us to hypothesize that these values do not correspond to an Ac-epitope-NO bond.

Production of NO_2^- was also determined to follow NO synthesis, and during use of NMMA the concentration of NO_2^- went from ~ 26 µM to a value on the order of 3. With normal macrophages no difference in concentration was found.

c) Neutralization of the cytostatic and cytotoxic effect of NO by the polyclonal Ab :

The cytostatic and/or cytotoxic role of NO-BSA formed in the co-cultures of the activated /T. musculi macrophages was followed for several days. We noted that the NO-BSA inhibited the multiplication of parasites and that use of the Ab of the invention blocked this effect. The same results were obtained when the supernatants containing NO-BSA were transferred into wells with normal / T. musculi co-culture macrophages.

Figures 7 and 8 represent the number of parasites counted every day for 4 days in the different culture wells:

- After incubation of the trypanosomes with the NO-BSA in accordance with the two procedures cited in "Materials and Methods" with or without normal rabbit serum or anti-BSA, the number of parasites remained almost stable and then decreased toward the 4th day. These results demonstrate the trypanostatic effect of NO transported in the form of "NO-protein" and the cutostatic and / or cytotoxic effect which was revealed by the decrease in the number of parasites.

- However, when the "T" and "C" Ab were added to the culture medium, the number of parasites increased from 104 to 106 parasites/ml between the days 2 and 4 of incubation. This same effect was also noted when NMMA was added to the activated macrophage culture. This demonstrates an inhibitor role of these molecules on the cytotoxic and cytostatic effect dependent upon the NO, which might be due to the NO-BSA or even to the nitrosylated epitopes (NO-Cys or NO-Tyr) formed in the nitrosylated BSA.

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Figure 7 represents inhibition of the cytostatic effect of the BCG macrophages on the *T. musculi in vitro* in the presence of NMMA (0.5 mM), ("T") or ("C") antiserum used at 1/100. The BCG trypanosome and macrophage co-cultures in normal medium and medium supplemented with normal rabbit serum or anti-BSA Ac,
5   were used as controls.

Figure 8 represents the cytostatic effect of supernatants containing NO-BSA from activated macrophages added to normal macrophages containing *T. musculi*. Inhibition of this effect in the presence of NMMA (0.5 mM), ("T") or ("C") antiserum used at 1/100. Absence of the inhibitor effect in the presence of normal rabbit serum or
10   anti-BSA Ab (1/100).

Each point on the curves of figures 7 and 8 is the result of 4 experiments.

3) Discussion.

The results above demonstrate the cytostatic and/or cytotoxic effect of
15   NO (in the form of NO-BSA) on the extracellular trypanosomes. They also demonstrate that the nitrosylated derivatives formed in the activated macrophage culture mediums, in the presence of BSA, are sufficiently stable to be detected by immunochemical methods.

The high specificity of the polyclonal antibodies of the invention permitted blockage of the "transported" NO effect in the trypanosomes. We can
20   conclude that one of the forms of NO transport during its cytostatic activity might be (NO-protein) or (NO-amino acid). We should also add that the immunochemical signal disappears when the NMMA is used or when the supernatant comes from normal macrophages with or without NMMA.

This work permitted *in vitro* evidence of BSA nitrosylation during its
25   exposure to NO produced at pH 7 from macrophages activated by the BCG. This nitrosylation is translated by a change in certain molecules which are cytotoxic for the parasites et carry nitrosylated epitopes, which are tyrosine and nitrosylated cystein, recognized by the polyclonal Ab of the invention.

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IV - PREPARATION OF MONOCLONAL ANTIBODIES DIRECTED AGAINST NO-CYS-GLUTARALDEHYDE CONJUGATES COUPLED WITH A CARRIER PROTEIN.

5   As already indicated, the nitrosylated derivatives intervene in a large number of physiological and pathophysiological bodily mechanisms (inflammation, septic shock, autoimmune diseases,... Moncada et al., 1991; Nussler and Billiar, 1993; Stevanovic-Racic et al., 1993; Anggard, 1994). It is therefore very important to obtain molecules capable of detecting and blocking these NO derivatives, which can destroy

10   pathogenic agents and block a major biological bodily function.

               The polyclonal approach previously reported demonstrated the possibility of blocking the cytostatic and cytotoxic effect of NO on T. *musculi*. However, the heterogeneity of the immune response does not permit these immune serums to perform adequately for complex studies focusing on a single epitope. To confirm the effect of

15   NO on the trypanosomes and to prevent non-specific attachments, a monoclonal approach was also developed. The Inventors therefore induced the monoclonal Ab directed against the NO-Cys-G-protein conjugate. This monoclonal Ab functioned firstly as a tool for *in vitro* detection of certain NO binding sites (such as cystein) in parasites, and secondly for *in vivo* inhibition of the development of inflammatory diseases such as

20   autoimmune encephalitis and experimental inflammatory arthritis in Lewis rats.

               1) Materials and methods.

               a) Immunogen synthesis:

               The immunogen used in the production of the monoclonal antibody is

25   Cys coupled with (G) on BSA or HSA and then nitrosylated by $NaNO_2$ in accordance with the protocol described in detail above.

               b) Method of immunization :

               - Intraperitoneal method (IP):

30   5- week old mice from the cosanguine line Balb/c (IFFA CREDO) were immunized using the IP method with 100 µg of immunogen as a Freund complete adjuvant for the first immunization. The other injections were done using the Freund incomplete adjuvant.

- Lymphatic pathway: plantar cushion (PC) :

With a solution of 1 mg/ml (in SPB) of conjugate, Balb/c mice got an injection in the (PC) every three days for 10 days (3 injections). Some received a fourth injection to increase the titer and avidity of the Ab response.

c) Obtaining monoclonal Ab directed against conjugated NO-Cys-G with the technique of lymphocytic hybridization and monoclonal selection :

The mice were immunized alternately with the NO-Cys-G-BSA and NO-Cys-G-HSA immunogens; when the titer, avidity and specificity of the Ab produced were deemed satisfactory, the selected mice were left for one month and then received a final intravenous booster shot 4 days before the lymphocytic hybridization.

Spleen cells (5×10^7) and myeloma SP2/O/Ag (2.5×10^7) (Shulman et al., 1978) were fused with polyethylene glycol 1500 according to an established fusion protocol. The technique used is derived from the one proposed by Kohler et Milstein (1975), improved by Lane (1985), and adapted to the laboratory for small molecules (Chagnaud et al., 1987; 1989a, b; 1990). Fused cells were then cultivated in a selective medium: RPMI 1640 enriched by L-glutamine (2 mM), in penicillin/streptomycin antibiotic and supplemented with fetal calf serum (10%) containing 50 µM of hypoxanthine and 10 µM of azaserine. After being cultured for 10 days, the wells containing the hybridomas were counted: The specific Ab producing clones were then screened with the ELISA test and sub-cloned according to the following stages:

- For the first screening, all the clones were tested on the following conjugates : NO-Cys-G-BSA, Cys-G-BSA and BSA-G. The anti-Ig mouse Ab of a goat were used at a dilution of 1/5000 to reveal positive clones, in particular the immunogen (NO-Cys-G-BSA).

- The positive clones were then sub-cloned (Sub-Cloning 1) to obtain a cell/well. After one week of culture the clones were retested on ELISA plates adsorbed by the conjugates cited above;

- After the first sub-cloning we studied the specificity of the positive clones. Thus, a dilution study was done in order to obtain a OD ~ 1 necessary for competition tests using the procedure described above;

- Clones which demonstrated a specificity for the NO-Cys-GBSA conjugate were sub-cloned for a second time (Sub-Cloning 2) and retested on nitrosylated or non-different conjugates : (NO-Cys-G-BSA; Cys-G-BSA; NO-Cys-BSA;

Cys-BSA; NO-Tyr-G-BSA; Tyr-G-BSA; NO-Tyr-BSA; Tyr-BSA; BSA-G; in addition
to NO-BSA). The results of the indirect ELISA tests and the competition tests enabled
us to choose the best clones;

- After these two sub-clonings verifying the monoclonality of the
5 hybridomas, the clones selected were stored in liquid nitrogen. Ab were mass produced
in ascite fluid (Potter, 1976) and the Ig were then purified through ammonium sulfate
precipitation.

2) Results.
10 a) Spectrophotometric analysis of the immunogen used for the
monoclonal approach :

Nitrosylation of the SH grouping of coupled Cys was studied using
spectrophotometry. Figure 9 represents the spectrometric analysis of the NO-Cys-G-BSA
immunogen and its structural homologue Cys-G-BSA based on wavelength. NO's
15 absorbency band for the NO-Cys-G-BSA conjugate was detected between the
wavelengths of 340 and 640 nm. The spectrum of the Cys-G-BSA conjugate which was
obtained before nitrosylation demonstrates an absence of absorbency between these two
wavelengths.

It should also be noted that the zone of absorbency obtained with NO-
20 Cys-GBSA is greater than the zone of the NO-Cys-BSA conjugate, despite the same
coupling relationship of the two conjugates. This may be due to the type of coupling and
therefore to exposure to the hapten accessible to NO.

Table 3 below demonstrates the molecular structure of the
NO-Cys-G-BSA and Cys-G-BSA immunogen:
25 Table 3

Cys-G-BSA (cysteinyl-glutaraldehyde)	$SH-CH_2-CH-NH-(CH_2)_5-NH-SAB$ $\quad\quad\quad\mid$ $\quad\quad\quad COOH$
NO-Cys-G-BSA (NO-cysteinyl-glutaraldehyde)	$NO-S-CH_2-CH-NH-(CH_2)_5-NH-SAB$ $\quad\quad\quad\quad\mid$ $\quad\quad\quad\quad COOH$

b) Evolution of the mouse immunizations:

Mouse immunized with the (IP) method:

Optimal dilution of the primary Ab is 1/8000. The evolution of two immune responses is noted : the significant one, directed against NO-Cys-G-BSA, was obtained after the third immunization. It is followed by a second, weaker one. This is the one directed against the hapten and carrier protein (Cys-G-BSA). Figure 10 represents the evolution of the antibody response during immunization of the intraperitoneally immunized mouse. The same dilution of the primary Ab was used for studying the avidity and specificity.

- Mouse immunized in the (PC) :

After the third immunization, the appearance of the response directed against NO-Cys-G-BSA was noted, and absence of recognition of the Cys-G-BSA. Figure 11 represents the evolution of the antibody response during immunization of the intraperitoneally immunized mouse. Le titer obtained after this immunization was insufficient to characterize the Ab (OD < 0.5), and a 4th immunization was done to improve titer and avidity.

c) Immunochemical characterization of the mouse immune serums :

- Study of the avidity and specificity of responses obtained in the two mice injected using two different methods of immunization :

The avidity and specificity of the anti-NO-Cys-G-BSA response was evaluated relative to the immunogen and its structural analogues, whether nitrosylated or not.

- Mouse immunized with the (IP) method :

Figure 12 represents the avidity and specificity of the anti-NO-Cys-G antibodies in the mouse (IP). The displacement curves were obtained after competition tests in the presence of the antibodies of each of the indicated conjugates. After incubation of the immunogen with the conjugated anti-NO-Cys-G diluted to 1/8000 the avidity of this antiserum was evaluated at half-displacement, and found to be 1.1×10^{-8} M. Relative to the other competitors used (Cys-G-BSA; NO-Cys-BSA; Cys-BSA; et la BSA-G) very weak recognition was found for Cys-BSA and BSA-G with, respectively, an IC$_{50}$ of 10^{-5} M and 5.6×10^{-6} M. Despite these crossed reactivities the NO-Cys-G-BSA is the best recognized conjugate.

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- <u>Mouse immunized using the (PC) method:</u>

Figure 13 represents the avidity and specificity of the anti-NO-Cys-G mouse antibodies (PC). The displacement curves are obtained after competition tests in the presence of the antibody of each of the indicated conjugates. After the fourth immunization, the Inventors noted in the mouse serum an increase in titer (to 1/70,000) and avidity of the conjugated anti-NO-Cys-G. The avidity of the Ab is calculated from the displacement curve obtained after incubation of the serum and immunogen in Figure 13. It is equal to 4×10^{-9} M.

Relative to study of the specificity, the molecules used (Cys-GBSA; NO-Cys-BSA; Cys-BSA and BSA-G) did not demonstrate a displacement of the OD values, signifying the absence of non-specific attachment with this Ab in these conjugates.

Through a comparison of these two types of polyclonal antibodies directed against the same epitope (NO-Cys-G), which differed from one another only by the method of administration of the immunogen, different values for titer and avidity were obtained. Furthermore, in the mouse immunized using the CP method, a quicker response was noted (at the end of 10-15 days) (Figure 11) which was more specific than in the mouse immunized using the IP method. For the latter, (Figure 10) the immune response was found within a timeframe well known for this traditional immunization method, varying between 20 and 30 days.

d) <u>Characterization of the monoclonal antibodies :</u>

The blood of the mice selected for this monoclonal approach was collected during sampling of the spleen. The signal on the corresponding nitrosylated conjugate was analyzed.

After 10 days of culture in a selective medium, yield of the fusion (number of wells with one or more clones) was 25 % for lymphocytic fusion of the mouse immunized using the IP method. However, for the fusion of lymphocytes from the mouse spleen immunized in the (CP), the yield was 97 %.

All the wells were tested and the yield (number of positive wells after an ELISA test) was approximately 85%. All the hybridomas selected recognized the immunogen. The background noise of the response (immunological signal on the non-nitrosylated conjugate / immunological signal on the nitrosylated conjugate) was lower than 10% in all cases.

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The clones that recognized the different conjugates were not selected.

- Avidity of the conjugated anti-NO-Cys-G monoclonal Ab :

The supernatant containing the anti NO-Cys-G monoclonal antibodies

5   was diluted to 1/5, and the ascite fluid to 1/30,000. These dilutions were chosen to yield an optimal OD $\simeq$ 1.0 to 492 nm. Figure 14 reports the avidity and specificity of the anti-NO-Cys-G monoclonal Ab. The curves were obtained after competition tests in the presence of the Ab and each of the conjugates indicated. These competition tests performed by incubating the NO-Cys-G-BSA immunogen in the presence of the

10   supernatant, demonstrates an avidity of 4 x 10^{-9} M. The ascite fluid used at 1/30,000 yielded an avidity of 2.5 x 10^{-8} M. This difference in avidity may be due to the presence, in the ascite fluid sampled from the mouse, of factors, proteins, or immune complexes which inhibit the specific responses.

15   - Specificity of the conjugated anti-NO-Cys-G monoclonal Ac:

The specificity of the monoclonal Ab was studied by incubating the supernatant from the hybridoma culture at a final dilution of 1/5 with conjugates structurally analogous to the immunogen: Cys-G-BSA ; NO-Cys-BSA; Cys-BSA; NO-Tyr-G-BSA; Tyr-G-BSA; NO-Tyr-BSA; Tyr-BSA; and BSA-G. At half-displacement,

20   only one Cys -BSA conjugate was recognized with a specificity of 4 x 10-6 M (Figure 14). These results demonstrate that the NO-Cys-G-BSA conjugate is the most easily recognized by these monoclonal Ab.

Despite the absence of competition with NO-BSA, indirect ELISA tests demonstrated that the monoclonal Ab recognizes the nitrosylated protein. The

25   supernatant (1/5) and ascite fluid (1/30,000) respectively yielded OD of 0.36 ± 0.085 and 0.54 ± 0.12. The results represent the average and standard deviation of three experiments.

- Determination of the isotypy of the monoclonal Ab produced :

30   Using a detection Kit for the mouse Ig (Mouse monoclonal antibody, isotyping kit, Sigma), we were able to determine the isotypy of the clone obtained: Ig G 2b.

3) Conclusion.

Analysis of the immunochemical characteristics of the conjugated anti-NO-Cys-G mouse Ab demonstrated the following:

5   - the immunodominant part recognized by the Ab is always the NO epitope;

- a slight structural difference between the immunogen and its structural analogues is taken into account by the Ab sites, confirming the elevated discriminatory potential of these immunoglobulins.

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These monoclonal Ab have a high degree of avidity and specificity; these immunochemical characteristics and the NO-BSA recognition permit them to be used in well defined biological models.

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V - DETECTION OF NITROSYLATED ANTIGENS AND PROTECTIVE ROLE OF MONOCLONAL ANTIBODIES.

In an immune response, the appearance of very reactive effector

20   molecules (oxygen derivatives, nitrogen derivatives,...) can change the antigenicity of the elements recognized by that response. Thus, the antigens carried by the microorganisms against which the body makes a Th1-type immune response with production of nitrogen derivatives can result in nitrosylated and nitrated derivatives. Since these nitrosylated antigens can entail the synthesis of antibodies, their presence and their potential role

25   should be studied. The fact that the nitrated derivatives are good, relatively well-known immunogens (Kofler et al., 1992; Maeji et al., 1992; Yuhasz et al., 1995), and the proven existence of anti-nitrotyrosine antibodies (Beckman et al., 1994a) led us to postulate the existence of such antibodies *in vivo* .

The first time, the inventors therefore looked for the presence of

30   nitrosylated antigens on the parasitic targets, with an immunocytochemical technique, using anti-nitroso-cysteine (polyclonal and monoclonal) and anti-nitroso-tyrosine (polyclonal) antibodies. The second time, since those nitrosylated antigens had been detected, they looked for the presence of corresponding antibodies in the serum of the trypanosomated animals. Trypanosomes are a favorite target of NO. In trypanosomoses,

indeed, a substantial increase in the number of macrophages and their activity can be seen. The cytostatic role *in vitro* and the induction of NO synthase found in the macrophages of infested mice indicate that NO could be involved in the effector mechanisms against the parasites. Moreover, trypanosomes circulating in their final host 5 (trypomastigote form) have a nonfunctional mitochondria, their energy depending on glycolysis. They are therefore much less complex targets of NO than the other pathogens or tumor cells, hence the interest in studying them.

While the cytostatic effect of NO on the natural trypanosome of the mice (*T. musculi*) and on the trypanosomes in the *brucei* group (*T. b. gambiense, T. b.* 10 *rhodesiense, T. b. brucei*), responsible for human and animal trypanosomoses has been well established *in vitro, in vivo* the effect of NO is much more complex. Indeed, NO participates in the mechanisms of immunosuppression by inhibiting the transformation and multiplication of lymphocytes, and it also induces apoptosis. The immunosuppressive role of NO is important in trypanosomoses because of the large 15 quantity of NO produced. That is why it is difficult with polyclonal and monoclonal antibodies to apprehend the study of nitrosylated molecules and a potentially protective effect linked to the injection of these antibodies. The third time, the potential role of the polyclonal and monoclonal antibodies in the invention *in vivo* was therefore studied in less complex experimental models, where the deleterious role of NO was already 20 strongly incriminated.

A - Immunocytochemical Approach.

1) Materials and Methods.

Peritoneal macrophages coming from mice activated with BCG (a source 25 of NO), and trypanosomes placed in a co-culture were used. The parasitic strain used was *T. brucei gambiense* (*T. b. gambiense* strain: "Féo" ITMAP/ 1893). These parasites were maintained after injection by the (IP) route (5 x 10^3/mouse) into normal mice (Noireau et al., 1989).

- Trypanosomes were cultivated in Petri dishes (35 mm) (Falcon Plastics) 30 with 2 x 10^6 parasites/ml/dish in a modified Mc Coy 5A culture medium (GIBCO) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM Hepes, 2 mM sodium pyruvate, 0.2 mM L-cysteine in the presence of peritoneal macrophages, activated or not (Albright and Albright 1980; Hirumi and Hirumi 1989; Vincendeau et al., 1985; Oka et al., 1988). NMMA was added to some dishes in a final dilution of 0.5

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mM. After 12 hours, the survival of the parasites was evaluated in the wells in the presence of normal or activated macrophages, with or without NMMA. Delipidated BSA was added to the culture medium in 4 mg/ml as an NO carrier. The supernatant containing the parasites was recovered after 12 hours to fix the parasites for the

5   immunocytochemical test.

- The trypanosomes were fixed on slides from which the grease was removed with organic solvents (ether-alcohol). The form of fixation most suitable for this protocol is done by cytocentrifugation (Shandon Elliott cytospin) at 100 g for 10 min. After fixation, the slides were dried, then after a saturation step with the buffer

10   TBS-BSA (1 g/l), the different primary antibodies were added. Incubation took place for 1 hour in a humid chamber at ambient temperature (Harlow and Lane, 1988). The anti-- NO-Cys-G (supernatant) monoclonal antibody was used to 1/50; the normal mouse serum used in the same dilution; the polyclonal anti-serums (rabbit "T" et "C") and a non-immunized rabbit serum were used in a final dilution of 1/10,000 to compare their

15   markings with that of the monoclonal antibody. The buffer alone was used on some slides as a negative control.

Incubation of the primary antibodies was followed by treating the slides with the secondary Ab marked with peroxidase in a dilution of 1/5,000 in SPB-BSA. Developing was then done with 3-3'diaminobenzidine tetrahydrochloride (DAB; Sigma)

20   at 60 %, H_2O_2 (0.03 %) in TBS.

2) Results.

25   The high-magnification (100X) immunocytochemical marking is shown in Figure 15 :

- the anti-NO-Cys-G monoclonal antibody:

- had very clear markings (immunoreactivities) in terms of trypanosomes co-cultivated in the presence of the activated macrophages (Photo 1);

30   -a much weaker marking was obtained in the co-culture of activated macrophages / trypanosomes, in the presence of NMMA (0.5 mM) (Photo 2);

- no marking was obtained on trypanosomes co-cultivated with normal macrophages.

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- A total absence of trypanosome marking was obtained when a normal mouse serum was used (Photo 3).

- No trypanosome marking was obtained when the secondary antibody (anti-Ig mouse marked with peroxidase) was used in the absence of a primary antibody.

These results affirm the positivity of the marking obtained in photo 1.

- Polyclonal antibodies "T" and "C" :

- antibody "C": photo 4 in Figure 15 shows an intensity very close to the one for the monoclonal Ab;

- the anti-NO-Tyr ("T") gives a positive marking with an intensity not as high as the two types of antibodies (monoclonal and polyclonal) directed against the epitope NO-Cys (photo 5);

- the use of the "T" and "C" Ab with normal macrophages showed a very weak marking of trypanosomes, compared to those obtained in the presence of the activated macrophages.

- The primary Ab of a normal rabbit showed an absence of marking (Photo 6).

- When the anti-Ig rabbit Ab marked with peroxidase were used in the absence of primary antibodies, no marking was observed.

3) Discussion.

These studies that were done show the cytostatic and cytotoxic effect of NO on extracellular parasites such as *T. b. brucei, T. b. gambiense* and *T. musculi* (Vincendeau and Daulouède, 1991; Vincendeau et al., 1992; Daulouède et al., 1994), like the cytostatic/cytotoxic effect of NO on *T. musculi* by nitrosylated BSA. This effect is amplified in the presence of a protein carrier in the culture medium and inhibited in the presence of a "C" antibody or NMMA, which shows the potential microbicide role of NO.

This study model of the cytotoxicity of NO is a simple, easy-to-show model compared to the use of other models, such as those of tumor cells. The latter have much more complex metabolisms than that of the trypanosomes, and it is more difficult to separate them from the macrophages than the trypanosomes that can be taken from the supernatants of co-cultures (macrophages / parasites).

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Moreover, *T. b. gambiense* has been used instead of T. *musculi* because the second strain is much more sensitive than the first to the action of NO or its derivatives. *T. musculi* need 48 hours for the NO, produced by macrophages activated with BCG to have a cytotoxic effect of around 90%. Other molecules, like suramine, which have an effect on the trypanosomes, require a carrier (BSA) to be fixed on the parasites (Collins et al., 1986; Lopez et al., 1992; Vansterkenburg et al., 1989). Since NO is much more reactive than suramine, it requires the use of a carrier providing transport, stability, salting out and transnitrosylation. This can take place by exchange of NO between the carrier protein and the proteins and/or amino acids of the trypanosomes, and on other parasitic sites not yet detected. The stability of the NO bond at these sites can vary from several "seconds" or even "hours" before it is oxidized into NO_2^- in the presence of O_2.

All these data point to using the *gambiense* strain as the NO target and delipidated BSA as the NO carrier. For the detection of nitrosylated derivatives such as NO-BSA or parasitic targets which have fixed NO, anti-NO-Cys-G monoclonal antibodies and polyclonal "T" and "C" Ab were used. To broaden the spectrum of detection of nitrosylated products, the use of antibodies directed against different NO epitopes and having different avidities makes it possible to be more precise in solving the problem posed. The immunocytochemical markers obtained with the polyclonal and monoclonal antibodies confirm the stability of the nitrosylated derivatives on the trypanosomes.

These results open up new prospects *in vivo* and *in vitro*, for pathologies involving the role of NO, such as neurodegenerative diseases, chronic and acute inflammatory diseases and inflammatory/autoimmunue diseases, such as multiple sclerosis and rheumatoid arthritis.

These new prospects are based on :

- evidence of antibodies developed against nitrosylated epitopes in parasitized mice;

- the development of experimental models in Lewis rats (experimental autoimmune encephalitis and adjuvant arthritis) which are respectively models of human pathologies: multiple sclerosis and rheumatoid arthritis. The purpose is to demonstrate *in vivo* the role of NO in the development of these two pathologies and neutralization of these harmful effects using the monoclonal antibodies in the invention.

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B - <u>Demonstration of antibodies directed against nitrosylated neoantigens</u>
in the serum of parasitized mice.

1) Materials and Methods.

Swiss mice were infested with three strains of parasites : *T. b. gambiense,*
T. b. brucei (strain: ANTAT 1.1) and *T. musculi*. Each mouse received 50,000 parasites
by the intraperitoneal route. Then the parasitemia was evaluated starting on the 3rd day
after infestation. Blood was then drawn from the mice on the 5th day (survival limit of
mice parasitized by *T. b. gambiense* and *T. b. brucei* and on the 21st day for the mice
infested with *T. musculi.*

Several nitrosylated conjugates were prepared for this immunoenzyme
study : NO-BSA; delipidated NO-BSA; NO-Cys-BSA; NO-Tyr-BSA; NO-Cys-G-BSA;
NO-Tyr-G-BSA and NO-Tryp-G-BSA. The non-nitrosylated structural analogues were
also used under the same conditions.

The serums were diluted to 1/1,000 and the secondary Ab were used in a
final dilution of 1/5,000.

2) Results.

- <u>Immunological response on nitrosylated conjugates :</u>

A study of the Ab directed against nitrosylated epitopes in the serum of
the trypanosomated mice was done on a population composed of: 15 mice infested with
T. musculi (group 1), 15 mice infested with *T. b. brucei* (group 2), and 15 mice infested
with *T. b. gambiense* (group 3). Serums from 15 normal mice were used as controls.

The averages of the OD obtained on different nitrosylated epitopes were
calculated for each group of mice mentioned above. Figures 16 to 22 show the OD
values for the normal mouse serum and the parasitized mouse serum. The OD obtained
on the molecules were corrected by subtracting the OD obtained on the non-nitrosylated
structural analogues which served as a blank on the ELISA test. These Figures 16 to 22
correspond respectively to the responses on the following epitopes: NO-BSA;
delipidated NO-BSA; NO-Cys-BSA; NO-Cys-G-BSA; NO-Tyr-BSA; NO-Tyr-G-BSA
and NO-Tryp-G-BSA. Each OD value is an average of 2 determinations; the bars in the
figures show the averages (calculated by the Mann-Whitney test) for the OD of each
group of mice.

- <u>NO-BSA and Delipidated NO-BSA :</u>

These nitrosylated proteins were recognized solely by the mice infested with the strain *T. b. gambiense*. The averages and the standard deviations obtained are 0.25 ± 0.029 (NO-BSA) and 1.1 ± 0.006 (delipidated NO-BSA).

- NO-Cys-BSA and NO-Cys-G-BSA :

A statistically significant immunological signal ($p < 0.0001$) was found only in the trypanosomated mice. The averages and the standard deviations obtained are the following:

Group 1 : 0.75 ± 0.39 (NO-Cys-BSA); 0.87 ± 0.37 (NO-Cys-G-BSA);

Group 2 : 0.60 ± 0.30 (NO-Cys-BSA); 0.30 ± 0.15 (NO-Cys-G-BSA);

Group 3 : 0.90 ± 0.34 (NO-Cys-BSA); 1.10 ± 0.41 (NO-Cys-G-BSA);

- NO-Tyr-BSA, NO-Tyr-G-BSA and NO-Tryp-G-BSA :

Likewise, an important, statistically significant immunological signal ($p < 0.0001$) was observed on these conjugates in the serum of trypanosomated mice, and absent in the serum of the control mice :

Group 1 : 0.72 ± 0.53 (NO-Tyr-BSA); 0.73 ± 0.27 (NO-Tyr-G-BSA); 0.60 ± 0.28 (NO-Tryp-G-BSA);

Group 2: 0.62 ± 0.30 (NO-Tyr-BSA); 0.60 ± 0.15 (NO-Tyr-G-BSA); 0.95 ± 0.33 (NO-Tryp-G-BSA);

Group 3 : 0.50 ± 0.26 (NO-Tyr-BSA); 0.93 ± 0.43 (NO-Tyr-G-BSA); 0.71 ± 0.22 (NO-Tryp-G-BSA);

For the control mice, the averages and the standard deviations obtained on the different conjugates are given in Table 4 below.

Table 4

NO-BSA	delipidated NO-BSA	NO-Cys-BSA	NO-Cys-G-BSA	NO-Tyr-BSA	NO-Tyr-G-BSA	NO-Tryp-G-BSA
0.02 ± 0.02	~0	0.03 ± 0.04	0.10 ± 0.03	0.02 ± 0.02	0.11 ± 0.02	0.11 ± 0.01

3) Discussion.

The results obtained show:

- a difference in immunological signals between the control serums and those of the parasitized mice.

- differences between the groups of parasitized mice, as well as between the different epitopes used. This could be due to the type of coupling that plays a role in the presentation of the nitrosylated epitope coupled to a protein by a peptide bond (carbodiimide) or by a "primary amine" type bond (G);

- These data confirm the involvement of NO in these parasitoses, which can be summarized as the result of an activation of the macrophages in the presence of the parasites followed by an expression of iNOS which releases a large quantity of NO for hours, even days. NO will therefore have its cytostatic or cytotoxic effect on the parasites by fixing itself to their protein targets and/or at the cell level of itself, thus forming different nitrosylated neoepitopes. These antigens will stimulate the immune system which will produce antibodies to these nitrosylated epitopes.

After having obtained these results in animals and because of the potential role of NO in the cytostatic and cytotoxic effect on intracellular and extracellular parasites, the Inventors studied patients with different parasitic pathologies (trypanosomosis, toxoplasmosis, amibiasis, hydatidosis, malaria) for changes in the immunological signs against nitrosylated epitopes. The results obtained showed a significant difference between the serums of patients with trypanosomosis or malaria and those of other patients and controls. For the first ones, the best immunological signs were obtained on the epitopes NO-Tyr-G and NO-Tryp-G. On the other hand, for the others, the immunological signals were very weak even on those two epitopes. The tests were done on 20 serums for each pathology.

C - Protective role of monoclonal antibodies in experimental autoimmune encephalitis and inflammatory arthritis

1) Experimental autoimmune encephalitis.

The purpose of the experimental autoimmune encephalitis (EAE) model is to reproduce the demyelinization observed in multiple sclerosis (MS). It is a demyelinizing pathology of the central nervous system (CNS) affecting the white matter. Myelin, a protective sheath of nerve fibers, is the target of the pathological process. Its deterioration is responsible for the appearance of MS. The origin of MS certainly depends on many factors: genetic, environment, autoimmunity and emotional stress (Antel and Cashman, 1991; Poser, 1992; Talbot, 1995). However, the exact

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etiopathogeny remains unknown. Several irregularities in immune response with cellular and humoral mediation have been described (Brochet and Orgogozo, 1987; Olsson, 1995). Hypotheses have been formulated concerning the autoimmune responses that have been found during the illness. They could cause an increase in the production of

5   NO by the macrophages/microglies, the smooth muscle cells and/or the endothelium of the CNS. Two mechanisms in cell destruction due to NO have been proposed (Sherman et al., 1992):

- Direct cytotoxicity by NO.

- Lesions due to the formation of peroxinitrite from the superoxide anion

10   and NO.

Other research has indicated the role of NO in MS (Offner et al., 1989; Villas et al., 1991; Koprowski et al., 1993; Van Dam et al., 1995; Weinberg et al., 1994), and the presence of the auto-Ab against the NO-Cys-G epitope in some patients (Boullerne et al., 1995).

15   To confirm all these data postulating the role of NO in MS and especially in EAE, the model was induced in Lewis rats by immunizing them with an encephalitogenic peptide of the myelin basic protein (MBP) of the guinea pig supplemented with *Mycobacterium tuberculosis* in some adjuvant. The goal of this approach is to show the involvement of NO in the creation of neoepitopes and hence in

20   the symptomatology of the disease, by blocking those epitopes with our monoclonal Ab which could have a preventive role. Changes in the immune responses to the nitrosylated and nitrated neoantigens in the serum of the rats were studied in parallel.

a) Materials and Methods.

25   Models of EAE in the Lewis rat were proposed by different teams (Panitch and Ciccone, 1981; Feurer et al., 1985; Levine, 1986; Polman et al., 1988). According to these authors, this induced model has the basic advantage of a high incidence of relapses and remarkable predictability.

30   - Encephalitogenic Antigen :

The animals were administered the encephalitogenic synthetic peptide (Peninsula laboratories) with a molecular weight of 1730 daltons whose sequence reproduces that of the fragment of guinea pig MBP between amino acids 68-84 whose chemical structure is the following:

Tyr-Gly-Ser-Leu-Pro-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn (SEQ ID NO.:___)
69   75   80   83

- Animals.

5   The work was done on Lewis rats, (IFFA CREDO) genetically susceptible to EAE. The rats were distributed in homogeneous lots by age between 6 and 7 weeks. Three groups of Lewis rats (average weight: 200 g) were formed. Each animal received subcutaneously in the plantar pads of each rear paw 0.05 ml of an emulsion of complete Freund adjuvant (ACP H37Ra, DIFCO) containing: 100 µg of peptide 68-84 of guinea
10   pig MBP in 0.05 ml of NaCl 0.9 %, plus 0.05 ml of ACF supplemented by 1 mg *Mycobacterium tuberculosis* (DIFCO).

The "Control Group" was composed of 7 rats. Each animal received only the emulsion indicated above.

The "Aminoguanidine" Group was composed of 5 rats. In addition to the
15   emulsion, after 4 days, this group also received a subcutaneous injection of a specific inhibitor of iNOS which is aminoguanidine (25 mg/kg), once a day for two days in a row (Reimers et al., 1994). The iNOS inhibitor was used to compare its activity with that of the anti-NO-Cys-G monoclonal antibody.

The "Ab monoclonal" Group was composed of 5 rats. In addition to the
20   emulsion, after 4 days, each rat received subcutaneously 5 mg/kg of monoclonal Ab purified by precipitation with ammonium sulfate (Mrabet et al., 1991; Lagier et al., 1992).

- Clinical Evaluation of Rheumatological and Neurological Symptoms :

25   The animals were observed clinically every day after administration of the emulsion.

The clinical tests were evaluated using criteria for :

- The appearance of arthritis which is characterized by the following score
: O = normal paws; 1 = average edema; 2 = more advanced edema; 3 = inflammatory
30   arthritis; 4 = inflammatory arthritis + difficulty walking; 5 = inflammatory arthritis + difficulty walking + ulceration; 6 = inflammatory and purulent arthritis + great difficulty walking + ulceration; 7 = inflammatory arthritis, purulent, and bleeding + great difficulty walking + ulceration.

- The behavior and tonus of each animal. The following clinical score was
35   used : O = normal; 1 = hypotonia of the tail; 2 = paralysis; 3 - paraplegia; 4 = tetraplegia.

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A score equal to or higher than 2 was required to affirm a neurological handicap (attack of EAE). If the score regressed, the animals were in the remission phase. On the other hand, when the clinical score reached a value at least equal to 2, the animals had a relapse (second attack).

- Immunoenzyme <u>Test</u> :

Blood was drawn once a week for 5 weeks after administration of the emulsion. The serums were tested at a final dilution of 1/500 under optimal test conditions. The signals were developed using anti-Ig rabbit antibodies of the rat marked with peroxidase (Sigma) diluted to 1/5,000. These serums were tested on the following conjugates :

- NO-Cys-G-BSA; Cys-G-BSA; and BSA-G: the last two are used to correct the OD obtained on the nitrosylated conjugate. The choice of NO-Cys-G-BSA lies in the fact that the monoclonal antibody used to block the clinical signs is directed against that epitope. The possibility of appearance *in vivo* of a neoantigen with the same spatial configuration and behavior identical to NO-Cys-G-BSA was studied.

- Recent work has shown the formation of nitrotyrosines at inflammatory sites (Kaur and Halliwell, 1994). To detect the presence of immunological responses to these epitopes in the serums of rats, NO_2-Tyr-BSA and the conjugated nitrosotyrorine (NO-Tyr-BSA) were used. Tyr-BSA was used to correct OD obtained on : NO_2-Tyr-BSA and NO-Tyr-BSA.

b) <u>Results.</u>

- <u>Development of EAE Model in Lewis Rats by Administration of an Encephalitogenic Peptide from the Guinea Pig:</u>

Each animal in the 3 groups in this study was examined clinically every day for 40 days.

- <u>"Control" Group (MBP alone) :</u>

Three days after immunization, the appearance of edema of the posterior paws was observed. Several days later, the rheumatological signs progressed toward ulceration, i.e., inflammatory arthritis with an average score of 5.

Around the tenth-twelfth days, the first neurologic signs appeared: hypotonia of the tail with difficulty walking, progressing to paralysis of the posterior paws. This condition was followed by a phase of remission, then relapse.

- Aminoguanidine Group :

Three rats out of five developed the disease. The same neurologic signs and signs of progressive arthritis as in the preceding group were observed in these rats. The other two rats developed only signs of arthritis. These results would indicate that aminoguanidine at a concentration of 25 mg/kg has relatively little effect.

- "Monoclonal Ab" Group :

The main purpose of this model is to show the role of NO in EAE. The monoclonal antibody in the invention developed against the epitope NO-Cys-G showed a protective role in the development of the disease, and showed that NO is involved in this animal model. The neutralization of the effect of NO by the Ab when the attacks appeared, is manifested by the following observations:

- total absence of neurologic signs: no hypotonia of the tail, difficulty walking or posterior paralysis;

- absence of the development of signs of arthritis : average edema (score 1) appeared at the immunization sites, with no inflammation or ulceration.

A follow-up of this group showed an absence of disease in these animals even after 40 days. There was therefore not just a delay in the appearance of the disease, but real protection. The results of this work open a new path for MS therapy. The demonstration of NO or certain epitopes induced during the disease in man will serve, for example, to understand the biologic and pathologic mechanisms that may trigger MS.

- Study of the evolution of Ab to nitrosylated and nitrated neoepitopes in rat serum:

The immunological signals directed against the conjugates NO-Cys-G-BSA; NO-Tyr-BSA; NO2-Tyr-BSA were studied. Figures 23 to 25 show the study of the evolution of the antibodies directed against the conjugates NO-Cys-G-BSA, NO-Tyr-BSA and NO2-Tyr-BSA, in the serum of rats (EAE). Figures 23 to 25 show, respectively, the evolution of three types of immune responses in the three groups "Control," "Aminoguanidine" and "monoclonal Ab."

The tests were done twice, and the results (OD) obtained are very similar between rats in the same group with non-significant differences. We therefore plotted all

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these curves after calculating averages and standard deviations for the OD obtained in all rats in the same group and on the same conjugate.

- "Control" Group :

For this group of animals, a correlation was found between the immune responses and the clinical response over time. Moreover, the immunological signal progresses identically for the three conjugates NO-Cys-G-BSA, NO-Tyr-BSA and NO2-Tyr-BSA. The OD obtained by the ELISA test on these conjugates are not very large (on the order of 0.125), but they can be considered like background noise because they are indicative of the presence of circulating Ab whose rate increases during attacks and, on the contrary, decreases during remissions (Figure 23).

- "Aminoguanidine" Group :

The anti-NO-Tyr-BSA and anti-NO2-Tyr-BSA responses are higher than those obtained in the preceding group (with OD between 0.25 and 0.50 starting in the third week after immunization). For the anti-NO-Cys-G-BSA response, the titer did not increase, but remained almost stable between the 1st and 4th week (Figure 24). Three rats developed several neurologic signs and the other two had very close results.

- "Monoclonal Ab" Group :

The immunological signals in this group are different from those of the first two groups. Figure 25 shows the changes over time in the anti-NO-Cys-G, anti-NO-Tyr and anti-NO2-Tyr responses. NO2-Tyr-BSA is the best recognized conjugate among the three (OD between 0.20 et 0.30 after the 3rd week). Moreover, there was a difference in recognition between the two epitopes nitrated-Tyr and nitrosylated-Tyr, caused by induced Ab. The anti-NO-Cys and anti-NO-Tyr signals remained stable between the 1st and the 5th week (OD ~ 0.125) except for a slight increase (after 1 week) in the anti-NO-Tyr, followed by a decrease.

These immunoenzyme tests made it possible to study rats that did or did not develop EAE for changes in the titers of antibodies potentially induced *in vivo* after the appearance of neoantigens, nitrosylated or nitrated. The serum drawn from the rats each week was limited. The number of tests and conjugates used was also.

It should be noted that on other ELISA tests done, an absence of discrimination of these Ab was noted between conjugate NO-Cys-BSA and conjugate Cys-BSA (carbodiimide coupling). The OD were identical on these two conjugates, which implies a non-specific bond and that the presentation of the NO-Cys at the end of

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a carbon chain (coupling G) probably gives that conjugate a configuration close to that of the epitope *in vivo*.

These results show that :

- Nitrosylated or nitrated derivatives (particularly the epitope NO-Cys) are responsible for the development of EAE in Lewis rats. These derivatives have spatial configurations *in vivo* identical to the conjugates NO-Cys-G-BSA; NO-Tyr-BSA; or NO2-Tyr-BSA absorbed on microtitration plates. This allows the Ab induced *in vivo* to show these artificial conjugates as being the derivatives against which they are directed.

- the monoclonal Ab has a protective role greater than that of aminoguanidine.

- These molecules produce their effects, on one hand, by neutralizing the harmful neoepitopes and, on the other, by stimulating the immune system. The latter therefore induces antibodies *in vivo* directed against these neoantigens generated after immunization of animals with an "immunogenic" type emulsion. This can explain the permanent increase in certain immunological signals in groups of rats that received aminoguanidine or the monoclonal antibody.

2) Experimental Inflammatory Arthritis.

The purpose of this model is to reproduce the inflammatory and rheumatological aspects present in rheumatoid arthritis (RA). This is a common autoimmune disease that starts in the form of inflammatory oligarthritis of the wrists and metacarpophalangeal joints (Bach, 1993). RA progresses with attacks occurring more or less frequently and interspersed with usually incomplete remissions. There seem to be several predisposing factors such as genetic, environmental and immune factors (Brostoff et al., 1993).

NO has recently been discovered as an effective element in this pathology (Stefanovic-Racic et al., 1993; Moilanen and Vapaatalo, 1995) and in other joint conditions (Stadler et al., 1991; Stefanovic-Racic et al., 1992). Its excess production may cause cytotoxic and cytostatic effects (Nussler and Billiard, 1993; Stefanovic-Racic et al., 1993; Anggard, 1994).

The presence of aromatic groups and amino acids nitrated with ONOO⁻ in biological fluids may be a marker for cell destruction *in vivo* (Kaur and Halliwell, 1994). In chronic inflammatory diseases such as RA (Halliwell et al., 1992), excessive production of free oxygenated radicals contributes to cell destruction. NO is also

recognized as participating in joint destruction (Farrell et al., 1992; McCartney-Francis et al., 1993; Stefanovic-Racic et al., 1993). Inhibition of NOS suppresses arthritis in mice (McCartney-Francis et al., 1993).

Recently, the production of NO has been measured indirectly in the form of nitrites in the scrums and synovial fluid of patients with RA (Grabowski et al., 1996). High levels of nitrites in patients compared to healthy controls show the role of NO as a possible mediating agent in rheumatism diseases.

Considering the bibliographic data indicating the role of NO in inflammatory diseases, particularly adjuvant rheumatoid arthritis (Ialenti et al., 1993; Oyanagui, 1994; Cannon et al., 1995), the Inventors studied the adjuvant arthritis model to determine whether the monoclonal antibodies directed against conjugated NO-Cys-G can inhibit the effect of NO in the induction of this pathology, and to see whether the epitopes or their derivatives responsible for the development of RA are related to those of EAE.

a) Materials and Methods.
- Adjuvant Arthritis:
All experiments were done on the strain of Lewis rats. Each animal received subcutaneously in the pads of each back paw 0.05 ml of an emulsion of ACF H37Ra containing : 0.05 ml of 0.9% NaCl, plus 0.05 ml of ACF supplemented with 1 mg of *Mycobacterium tuberculosis*.

Homogeneous lots of rats were formed. They were subject to the same conditions as those used for the EAE model. Four groups of 5 rats, age 7 weeks (average weight: 200 g) were formed.

- "Control" Group : each rat in this group received only the emulsion mentioned above.

- "NIS" non-immune mouse serum group: each rat received the emulsion, and after 4 days a subcutaneous injection of 5 mg/kg of immunoglobulins (precipitated with ammonium sulfate) of normal mice. The injection was given once a day for two days.

- "Aminoguanidine" Group : each rat received the emulsion, and after 4 days a subcutaneous injection of aminoguanidine (25 mg/kg) once a day for two days.

- "Monoclonal Ab" Group: each rat received, in addition to the emulsion and after 4 days, 5 mg/kg of anti NO-Cys monoclonal antibody (precipitated with ammonium sulfate) by the subcutaneous route.

5   - Clinical Evaluation of Arthritic Symptoms:

Special attention was given to detecting an arthritic joint affliction. The inflammatory condition of the paws was evaluated in the following way: edema of the paw (not interfering with walking); large paw; edema of the paw interfering with walking or "inflammatory" paw.

10   Based on the arthritic score already mentioned above, it was possible to follow the rats in these 4 groups for the progression of clinical signs over time for 40 days after immunization.

- Immunoenzyme Test :

15   The Inventors studied the rat serum for the presence of antibodies to nitrosylated or nitrated epitopes that may appear *in vivo* during the development of this model. We therefore tested the presence of immunological signals on the following conjugates NO_2-Tyr-BSA; NO-Tyr-BSA; Tyr-BSA; NO-Cys-BSA; Cys-BSA and BSA-G for the same reasons given in the preceding model. The conditions on the ELISA

20   test were also the same.

b) Results.

- Comparative Study of Clinical Results :

"Control" and "NIS" Groups: The rats in these two groups developed the

25   typical signs of articular arthritis, which translate into :

- the appearance of edema in the plantar pads of the posterior paws two days after immunization;

- these rheumatological signs progressed with time, toward large paws with purulent ulceration;

30   - after around 10 days, difficulty walking;

- some rats had joint deformations on their posterior paws.

In conclusion, these two groups showed no differences. The clinical scores for most of these ten rats were 5, 6 and 7. Non-immunized mouse serum injected

4 days after administration of the antigen had no effect on the aggravation of the disease or on the protective effect.

"Aminoguanidine" Group : Three rats developed edema without difficulty
5   walking, then had scores of 4 and 5. The other two rats had scores of 6 and 7.

"Monoclonal Ab" Group: the results obtained with these rats may be described in the following way:
        - appearance of only average edema in all rats at the end of the first week;
10   - absence of progression to the ulceration stage;
        - no difficulty walking: the rats maintained their ability to lean normally
on their back paws;
        - only one rat had an inflammatory stage with no difficulty walking, or the presence of bleeding or purulent arthritis which was observed in the "Control" and
15   "NIS" groups.

In conclusion, NO or one of its derivatives, which can be NO-Cys, peroxinitrite, nitro-tyrosine or other oxygenated reagents, has a role in the development of the model of articular arthritis and hence in the inflammatory processes.
20

        - Study of the Progression of Ab Developed Against Nitrosylated and
Nitrated Neoepitopes in the Serum of Rats :
        Figures 26 to 29 report the study of the progression of the antibodies directed against the conjugates NO-Cys-G-BSA, NO-Tyr-BSA and NO_2-Tyr-BSA in the
25   serum of rats (RA), Figure 26 for the "Control" group, Figure 27 for the "NIS" group, Figure 28 for the "Aminoguanidine" group and Figure 29 for the "Monoclonal Antibody" group. Each point represents the average of the standard deviation for OD obtained in all the rats.

30   - "Control" and "NIS" Groups :
        The serums of the ten rats drawn for 5 weeks were tested on conjugates NO-Cys-G-BSA; NO-Tyr-BSA; NO_2-Tyr-BSA, and on the corresponding non-nitrosylated conjugates. Figures 26 and 27 show the "Control" and "NIS" groups, respectively, with the progression over time of the antibodies induced against two

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epitopes: NO_2-Tyr-BSA and NO-Tyr-BSA. These results represent the average on two tests. The OD obtained in each group are equivalent on all conjugates tested, each point represents the average and the standard deviation of the OD obtained with the 5 rats in the same group for the same conjugate.

5   The OD obtained for these two conjugates vary between 1 and 2 between the 2nd and 5th weeks. For conjugate NO-Cys-G-BSA, the signals are very weak (OD ~ 0.1).

- The "Aminoguanidine" and "Monoclonal Ab" Groups:

10   The immunological signals revealed in the 10 rats in these two groups are equivalent between the rats in the same group and on all conjugates tested. The curves show the average of two tests, and the results are analyzed in the same way as the preceding groups. As in the other groups, the signals remain very weak for conjugate NO-Cys-G-BSA.

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       For "Aminoguanidine": (Figure 28) anti-NO-Tyr is the highest response; the OD are between 1.5 and 2 (between the 2nd and 5th weeks). The anti-N02-Tyr response is as large; note an increase in signals between the 1st and 2nd weeks. They stabilize until the 3rd week, then increase slightly toward the 4th week.

20

       For the "monoclonal Ab" group (Figure 29): note that in these animals, the anti-NO-Tyr response is greater than the anti-N02-Tyr. But note also that a plateau was found for the two types of Ab, and this was between the 3rd and 5th weeks. This may be due to a cross reactivity between these Ab, since the two responses progressed,

25 then stabilized in the same periods. Moreover, the anti-NO-Tyr and anti-N02-Tyr levels in the serums of these rats are slightly less large than those obtained for the rats in the "NIS" and "Aminoguanidine" groups. On the other hand, the curve representing anti-NO-Tyr has the same appearance and the same amplitude as the one in the "Control" group.

30   Due to the size of the anti-NO-Tyr and anti-N02-Tyr responses, other tests were done to look for anti-NO-Tyr-G-BSA Ab. Thus, some OD obtained under the same ELISA test conditions were almost negligible in the four groups. These results mean that the presentation of the epitope is essential. A difference in the spatial

configuration between these different nitrosylated epitopes is surely present *in vivo*. It is essential to induce an immune response.

In conclusion, these immunoenzyme tests showed no significant difference between the four groups. An increase in the titer of antibodies induced against nitrosylated and nitrated epitopes was found in each sample. For the "monoclonal Ab" group, effective, but partial protection of rats was observed; on the other hand, the immunological responses obtained in the animals in this group were identical to those in the other three groups.

10   c) Discussion.

Production of circulating antibodies was found in the serum of animals on the ELISA test in the two models. For EAE, recent work showed the presence of the anti-NO-Cys-G antibody in the serum of rats which developed that pathology (Boullerne, 1996, University thesis). The above work confirms the involvement of the neoepitope (NO-Cys), among other factors triggering neurologic symptoms, in this experimental disease and hence the presence *in vivo* of antibodies recognizing this epitope. The use of monoclonal antibodies directed against this same epitope is therefore essential to catch it.

For RA, no work has shown the involvement of the epitope NO-Cys in the development of that disease. But cysteine (Stamler et al., 1992b) is a target of nitrosylation, with formation of thionitrites. Moreover, nitration of tyrosine, by ONOO⁻ for example, (Beckman et al., 1994b; Van Der Vliet et al., 1994) gives nitrotyrosine which has been shown in inflammatory processes. Transnitrosylation processes may also take place *in vivo*, between different molecules carrying thiol residues accessible to NO (Scharfstein et al., 1994) which produces several nitrosylated products. In the adjuvant RA model, NO and the superoxide anion are considered factors for the induction and development of this pathology (Ialenti et al., 1993; Oyanagui, 1994; Cannon et al., 1995). This experimental model was induced in rats injected with mycobacterium, in the presence or absence of aminoguanidine or the monoclonal antibody directed against an NO-conjugated epitope to compare their protective role.

The protection of rats with Ab to the development of the disease in EAE rats is 100%. The monoclonal Ab have a less important inhibiting role in the RA model, suggesting the intervention of factors other than NO-Cys in triggering the disease. The

good tolerance of the monoclonal mouse Ab by the rats should also be noted due to the short immunological distance between the mouse and the rat.

Moreover, the immunological responses found in the serum of all the animals against the conjugates NO-Cys-G-BSA; NO-Tyr-G-BSA and NO_2-Tyr-BSA,

5   make it possible to follow up and compare not only the clinical signs, but also the appearance of those signals and increases in them.

The monoclonal Ab in the invention made it possible to show the role of NO in the development of EAE where the results obtained were very satisfactory. On the other hand, the use of these Ab in rats developing adjuvant RA is less effective, which

10   limits the use of these monoclonal Ab to the epitope against which they are directed.

VI - CONCLUSIONS.

The possibility of obtaining the induction of a specific immune response

15   directed against nitrosylated epitopes by using synthesized immunogens resulted in studying the possibility of that induction under "natural" conditions. The presence of Ab directed against nitrosylated epitopes in parasitic conditions where, on one hand, the quantities of Ab produced are considerable and, on the other, the involvement of nitrogen monoxide has been shown, was studied.

20   In these conditions, there is indeed an increase in the level of IFN-γ, evidence of different roles of NO and production of nitrites by peritoneal macrophages. In the case of the research work showing *in vitro* the cytotoxic role of NO on intracellular parasites like *Leishmanies*, the activated macrophages have been identified as cytotoxic to those parasites (Murray et al., 1983; Hall and Titus, 1995). More recently,

25   the nitrogen oxides, particularly NO released by the activated macrophages, have been considered essential products for their intracellular cytotoxicity against *Leishmanies* (Liew et al., 1990; Reiner and Locksley, 1992). In humans, Vouldoukis et al. have also demonstrated this effect in the presence of activated human monocytes (1995). They showed that ligation of CD23 with the IgE-anti-IgE complex, or with the anti-CD23

30   monoclonal antibody induces synthesis of NO. Moreover, there was a generation of different cytokines by the human monocytes/macrophages. Thus, ligation (IgE-anti-IgE)/CD23 induces a cytotoxic effect on intracellular parasites: *Leishmania major* in human macrophages via induction of the L-arginine/NO route. This was demonstrated by an increase in NO2-, and by the blockage of that effect by NMMA.

Likewise, activation of these human macrophages by IFN-γ/TNFα induces the same leishmanicidic activity inhibited by an anti-TNF-α.

On the other hand, the inventors showed the cytostatic effect of human monocytes on extracellular parasites (T. gambiense). In the presence of the monoclonal Ab (anti-NO-Cys-G) or NMMA, this trypanostatic effect was inhibited, indicating then the role of NO. All these results show the possibility of activating human monocytes to induce a cytostatic and/or cytotoxic effect on intracellular or extracellular microorganisms.

Research work done in vivo has also shown the role of NO in the defense of the host against leishmaniosis. An injection of NMMA in mice resistant to this parasitosis inhibits endogenic production of NO and exacerbates the lesions (Evans et al., 1993). More recently, it has been shown that mutation of the gene for iNOS in resistant mice makes them susceptible (Wei et al., 1995). In trypanosomosis, there is a "Th1" type response directed against the parasitic antigens (Schleifer et al., 1993). The presence of products that are parasitic in origin triggers the production of IFN-γ, TNF-α, and iNOS causes hyperproduction of NO (Olsson et al.,1991; Sternberg et Mabott, 1996). In addition to its antiparasitic role, the NO produced participates in the immunosuppression observed during trypanosomosis.

The chronology and the production site of NO can be decisive in the global effect linked to NO observed in trypanosomoses. In mice (C57 BL/6) resistant to Plasmodium chabaudi SA, there is premature expression of iNOS in the spleen, correlated with resistance to disease. In susceptible mice, there is late expression of iNOS in the liver (Jacobs et al., 1995). The same strains of mice are "resistant" (C57 BL/6) and susceptible (BALB/c) to Leishmania major (Evans et al., 1993; Wei et al, 1995) and to T.-musculi (Albright and Albright, 1981). Protection against Leishmania major is correlated with production of NO.

In trypanosomoses caused by trypanosomes from the brucei group, very marked immunodepression can be seen. Inhibition of NO synthesis prolongs the survival of the trypanosomated animals (Sternberg et al, 1994). The cytostatic and/or cytotoxic role of the NO produced by the macrophages on T. musculi has thus been demonstrated. By using different nitrosylated conjugates, it was possible to detect Ab in the serum of trypanosomated mice directed against several nitrosylated epitopes. The presence of these Ab deserves to be emphasized on several scores. First of all, they appear naturally in the course of a Th1-type immune response, which causes production of NO, whose

attachment to the antigens causes configurational changes recognized by the immune system.

In different parasitic diseases, the "induced" Ab have the role of "marker" of the parasitosis, and little effectiveness against the pathogenic agent; in some cases, they mask the parasites or block the action of other Ab that are more effective than they are. The characterization of these Ab is therefore of interest. Also, demonstration of these Ab at the different stages of a disease may supply information on the successive types of immune responses provided. Thus, in some diseases, protection is provided by the succession of a "Th1"-type response and a "Th2"-type response. Could the appearance of antigens modified by NO and its derivatives trigger the induction of a protective-type immune response?

Whatever their role, detection of nitrosylated antigens may prove highly interesting. Polyclonal and monoclonal Ab may be a great help in detection and may prove to be of crucial interest. Thus, some antinitrotyrosine Ab allow detection of nitrotyrosine, whose appearance is associated with the presence of peroxinitrite (Van Der Vliet et al., 1994; Ischiropoulos and Al-Mehdi, 1995; Crow, 1996). This Ab therefore makes it possible to learn the situation where peroxinitrite is synthesized and to detect the targets of the disease. Nitrotyrosine has been displayed using immunological techniques in the coronary vessels during atherosclerosis (Beckman et al., 1994a). Moreover, in patients suffering from RA, it has been detected in the synovial fluid (Farrell et al., 1992). But despite a correlation between nitration and development of a pathology, it remains to be studied whether nitration of the tyrosines at the protein level causes primary lesions which trigger inflammation, or if it is secondary to the inflammatory processes. Indeed, peroxinitrite reacts in a complex way with different biological molecules causing hydroxylation, fragmentation of DNA, oxidation of the iron-sulfur centers of the proteins, etc. and especially the formation of nitrotyrosine which is the most marked trace of peroxinitrite. This nitrotyrosine thus formed induces tissue lesions via several mechanisms (Beckman et al., 1994a): 1) alteration in the phosphorylation of tyrosine; 2) alteration in protein functions due to the introduction of negative charges on hydrophobic sites of tyrosine, which could alter the protein configuration; 3) initiation of autoimmune processes by the presence of nitrophenols such as nitrotyrosine, which is recognized as very antigenic (Kofler et al., 1992; Maeji et al., 1992; Mizutani et al., 1995; Yuhasz et al., 1995).

From these data showing the role of nitrotyrosine in inflammatory and destructive processes, it is possible to deduce that the Ab directed against the different elements derived from NO can also permit their characterization and a knowledge of their properties. For example, the well-known role of the thiols, the S-nitrosothiols and S-nitrosoalbumin in vasodilatation *in vivo* and *in vitro* (Stamler et al., 1992 c, d; Keaney et al., 1993; Simon et al., 1993). The anti-NO-Cys Ab made it possible to establish an immune function carried by albumin. Note here that NO-BSA can serve as a reservoir of NO in the plasma, from which NO can be transported to the intracellular environment by a transnitrosylation mechanism. After its transport, NO has access to its intracellular targets, such as guanylate cyclase or the hemoproteins, which results in a physiological activity that can become pathological in the case of alteration at the level of transnitrosylation.

The NO-BSA form made from a biological source (macrophages) has been widely used, but the first time, other nitrosylated molecules synthesized with an acid pH from an NO donor were used. This made it possible to develop polyclonal and monoclonal Ab. The second time, the presence of nitrosylated molecules was detected using these Ab. These nitrosylated derivatives were synthesized in co-cultures: activated macrophages/BSA or activated macrophages/BSA/parasites. Detection was done not only by the immunoenzyme technique, but also by the revelation of the cytotoxic role of NO on extracellular parasites. This effect of NO is amplified in the presence of the carrier protein, BSA, and has been neutralized in the presence of the Antibodies in the invention. The activated macrophages produce NO, but can also produce other oxygenated derivatives like HOCl (De Violet et al., 1984; Vincendeau et al., 1989), H_2O_2 (Vincendeau et al., 1981), and above all $O_2^{-\circ}$ The question is whether the presence of this anion is necessary for the formation of nitrosylated albumin.

$O_2^{-\circ}$ or another derivative of oxygen (H_2O_2, HOCl) can be the element reacting with NO. Indeed it is known that murine neutrophiles and macrophages simultaneously produce NO and H_2O_2 as well as other oxygenated reactive species that participate in cytotoxicity (Pacelli et al., 1995).

Indeed, it is known that nitrogen and oxygen derivatives can also interact to form derivatives having different functions. Neutralization of these functions by selective blocking of one molecule or another can prove very instructive. Moreover, it should be noted that when a chemical donor of NO ($NaNO_2$) is used, an acid pH was

necessary for nitrosylation. On the other hand, in the culture medium, the pH is neutral. We can respond to this question by considering Kharitonov's data which shows that nitrosylation can take place *in vitro*, in an oxygenated medium and at pH 7 by means of N_2O_3 (Kharitonov et al., 1995), even if that nitrosylation is less important than that of

5   Cys or glutathione, for example. But to answer this question, it will be necessary to use trappers for oxygenated derivatives like SOD, catalase and taurine, which will make it possible to study nitrosylation *in vitro*, at a neutral pH.

The nitrosylation of BSA *in vitro* and the corresponding cytotoxic effect require clarification of an important point concerning the experiments done under

10   slightly different conditions :

- Under the first conditions, the trypanosomes and BSA were incubated together. The activated macrophages will produce NO, but also many other free radicals. Thus, oxygenated derivatives, toxic or not, will be formed. This enables us to postulate that, during incubation, these derivatives could act "in competition" with NO on the

15   trypanosomes to mask and/or interfere with its toxic effect.

- Hence the interest in the second experimental conditions, where BSA was placed in the presence of activated macrophages to be nitrosylated. Then, the NO-BSA formed was transferred to media of normal parasite/macrophage co-cultures which produce no free radicals or very few. Under these conditions, it can be assumed that the

20   effect of the NO-BSA added to these cultures, poor in free radicals, is due to NO and not to other oxygenated derivatives.

- The results obtained in these experiments were confirmed using an anti-BSA Ab. When this antiserum was used, the trypanocidic effect of NO-BSA persisted under the first and second conditions. This indicates that the neutralizing effect,

25   observed in the presence of the "T" or "C" Ab, is proper to the latter despite their polyclonal character.

It should also be added that the second conditions also made it possible to show the absence of a "stimulating" or "inhibiting" effect of the immune complexes that could be formed in the macrophages. Indeed, the use of normal macrophages (not

30   expressing iNOS), in the presence of polyclonal Ab, trypanosomes and some supernatant containing nitrosylated BSA, showed results equivalent to those obtained under the first conditions. This shows that whatever the influence of immune complexes on activated macrophages, NO production from those cells (expressing iNOS) was not changed. Thus, we can deduce that that under these two conditions, the cytotoxic effect of NO on

T. *musculi* is due particularly to the formation of NO-BSA. It therefore exerted this effect either by releasing NO which is fixed on its targets, or by a mechanism of NO exchange between the BSA and the amino acids or parasitic proteins.

Evidence of the formation of nitrosylated derivatives in *vitro* by immunoenzyme tests or by the cytotoxic effect led to a search for the NO fixation sites in these parasites. This search started using the immunocytochemical technique :

- The best results were obtained with activated macrophages. Polyclonal and monoclonal Ab were used as tools for marking NO fixation sites in the parasites. These results were very reproducible, which was not the case when chemically synthesized NaNO$_2$ or NO-BSA was used. With these molecules, the half-life of the trypanosomes did not exceed 10 min and their form was not kept. We explained this fact by the presence of excess NO released by these chemical donors, which had an intense action on the parasites, translating into their lysis.

- The use of controls gave weak markings or a total absence of immunoreactivity. This confirmed the positivity of the markings obtained in the presence of activated macrophages (without NMMA) with polyclonal and monoclonal Ab used as the primary Ab.

These specific Ab of nitrosylated epitopes showed the presence of at least nitrosylated cysteine and tyrosine residues. These were recognized by their corresponding Ab. This immunoreactivity could be explained either by the fixation of nitrosylated BSA to the parasite surface, or by transnitrosylation mechanisms between that molecule and the parasitic antigens carrying a thiol residue. Their nitrosylation could therefore be the origin of the cytostatic effect mediated by NO-BSA. Indeed, some of the many studies have demonstrated the role of NO in modulating the activity of certain proteins fixing on their thiol residue (Stamler et al., 1994) which permits regulation of certain cellular mechanisms. Among them are soluble guanylate cyclase (Stamler et al., 1992a); the receptor NMDA (Lipton et al. 1993); protein kinase C (Gopalakrishna et al., 1994); p21raS (Lander et al., 1995); GADPH (Mohr et al., 1996) and the transcription factor OxyR (Hausladen et al., 1996). The transnitrosylation of NO from BSA to thiols of the biological molecules depends on the reactivity of those molecules, that of BSA and the stability of the S-nitrosothiol formed (Scharfstein et al., 1994). Thus, to identify these nitrosylated molecules detected in the trypanosomes, experiments by immunoimprints using NO gas or NaNO$_2$ for the nitrosylation of the parasitic proteins are conceivable. These experiments allow the detection of protein sites

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that were accessible to the phenomenon of nitrosylation *in vitro*. The results obtained are very preliminary and not very satisfactory. Indeed, nitrosylation under well determined conditions could entail better accessibility to the corresponding Ab. Note that this hypothesis has already been cited for nitrated derivatives (Crow, 1996).

5   This protocol can be optimized using activated macrophages as NO donors. Mechanisms acting during activated parasite/cell contact, aiding in nitrosylation and structural modification of the protein targets can be envisaged. After their nitrosylation from chemical donors of NO, the proteins may be better recognized by the Ab than when they are nitrosylated chemically. It is important to note here that a

10   significant difference between nitrosylation produced by enzymatically synthesized NO and that produced by chemical donors of NO has been demonstrated (Ignarro et al., 1993). Finally, there are probably other sites of nitrosylation on the parasites, other amino acids (lysine, tryptophane, etc.), iron-sulfur centers of certain enzymes, etc.

       After this work *in vitro*, the pathological role of NO was studied *in vivo*.

15   NO was demonstrated in many pathologies (Bagasra et al., 1995; Moilanen and Vapaatalo, 1995; Grabowski et al., 1996). For example, in the case of septic shock, the expression of iNOS in the vascular system results in vasodilatation, a reduction in vasoconstrictive activity, hypotension, and tissue destruction (Petros et al., 1994). But, these changes in the vascular system are not strictly due to free NO. Many *in vivo* and *in*

20   *vitro* studies have shown that S-nitrosylated derivatives are vasodilating compounds, inhibitors of platelet aggregation and regulators of blood flow and pressure. The most studied are the S-nitrosocysteines, S-nitrosoalbumin, and even S-nitrosylated hemoglobin (Stamler et al., 1992b, c, d; Keaney et al., 1993; Jia et al., 1996). These molecules, characterized by a longer half-life than that of NO (Stamler et al., 1992c),

25   will participate in the aggravation and evolution of the pathological processes in septic shock. They are probably due to an increase in the production of GMPc from guanylate cyclase, and to the phenomenon of NO exchange between thiols of proteins and low-molecular-weight thiols. This triggers a cascade of events prolonging the harmful effect of NO, especially on the vascular level.

30   It should also be recalled that in other pathological conditions, excessive production of NO *in vivo* can cause varied neurological problems. For example, some work has made it possible to establish that some populations of neurons are resistant to the toxic effects induced by the release of excitor amino acids like glutamate, while they produce toxic quantities of NO (Beal et al., 1986; Koh et al., 1986; Koh and Choi,

1988). These neurons seem to be preserved also in pathologies like Alzheimer's disease or Huntington's chorea where the role of NO has been demonstrated (Ferrante et al., 1985; Meldrum and Garthwaite, 1990; Olney, 1991). More recently, NO has been identified as an effector element in neurological problems observed in patients with

5   acquired immune-deficiency syndrome (Bukrinsky et al., 1995; Lipton and Gendelman, 1995). NO is considered necessary but not sufficient to induce cell death. It can react with other free radicals like the superoxides, causing the formation of peroxinitrite, a particularly toxic chemical (Beckman et al., 1990; Radi et al., 1991).

    NO is also one of the mediators involved in different "autoimmune"

10   diseases. Thus, for example, it could be involved in the destruction of the b cells in the islets of Langerhans and could entail the appearance of diabetes (Welsh et al., 1994; Lindsay et al., 1995; McDaniel et al., 1996). Moreover, synthesized NO can attach itself to some amino acids (especially cysteine and tyrosine), thus causing nitrosylation and a modification in the proteins or enzymes at the tissue level. These proteins of themselves .

15   can therefore become, under pathological conditions, molecules that are foreign to the organism, inducing antibodies in humans, as in the case of multiple sclerosis and inflammatory arthritis. Nitrosylation of antigens and the appearance of the corresponding Ab would be a natural phenomenon, eventually heightened by overproduction of NO. The role of these antigenic nitrosylated derivatives could be the destruction of the target

20   cells that carry them. Thus, nitrosylation of microbial antigens could be accompanied by those of the antigens of themselves. The Ab produced, besides their various roles on the exogenic antigens, could also act on themselves. Depending on the quantity produced, the biological properties associated with the isotypy of the Ab, and its affinity, different results could be observed: destruction of cells, masking of epitopes entailing protection,

25   etc...

    From an experimental standpoint, the involvement of NO in experimental inflammatory and immune pathologies induced in the Lewis rat is now well established. Several authors have reported by different approaches that NO could have an important role in these degenerative processes, one touching on the central myelin, another on

30   articulation and its synovial membrane.

    Two animal models were developed within the framework of the invention. The first which mimics the neurological evolution of MS and the second which consists of a model of adjuvant inflammatory arthritis. The role of NO and its nitrated derivatives in the inflammatory processes and tissue destruction has been

demonstrated (Halliwell et al., 1992; Winyard et al., 1992; Morris et al., 1995). Next, some circulating Ab that appear prematurely after the induction of the experimental models were shown. These immunoglobulins recognize three antigen targets carrying NO or NO_2 NO-Cys-G, NO-Tyr and NO_2-Tyr. Their presence could be a sign of antigen

5   stimulation associated with hyperproduction of NO. On the other hand, an analysis of the physiopathological, clinical and immunological processes confirms an increase in the production of NO. It could be located mainly in lesions and undoubtedly related to macrophage stimulation. But are the endogenic targets: Cys and Tyr, potential transporters of NO, key elements in the physiopathogeny? The therapeutic effects of the

10   monoclonal Ab anti-NO-Cys-G emphasize the potential role played by the epitope NO-Cys. Recognition *in vivo* of this target entails the abolition of the symptoms of EAE and considerably reduces inflammatory arthritis. The antigen NO-Cys thus appears, under these conditions, as a key step leading toward myelin destruction for EAE and inflammation of the synovial membrane in arthritis.

15   The presence of Ab in the serum of experimental animals and the protective role of injected Ab indicate that neutralization of the epitope NO-CysG plays an important protective role. The absence of protection in animals not injected, despite the presence of the Ab, indicates that (i) the Ab produced have too little affinity or there are too few of them or they are produced too late. (ii) The Ab produced do not have the

20   same functions or may be another isotype than the Ab injected. Isotypic determination, immunochemical study of the Ab produced, their purification, then their injection into experimental animals will make it possible to answer these questions.

      Finally, the two animal models used made it possible to obtain important results. Taking EAE (the experimental model of MS in humans), for which almost total

25   protection was noted, the results obtained constitute a therapeutic approach to MS.

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